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This is a final report of an IDEA Award to study the molecular mechanisms driving acquired antiestrogen resistance. We have identified several genes associated with resistance to ICI 182,780 (Fulvestrant. Faslodex). Additional studies with NFkB reversal with parthenolide have now been completed and submitted for publication (accepted pending minor revisions). Further functional studies on the role of IRF-1 using a dominant negative construct have been published (Bouker *et al* Cancer Res 64:4030, 2004) and we have collaborated on an additional study that has identified a new estrogen receptor variant in our hormone resistant cells (Han *et al.* J Mol Endocrinol 32: 935, 2004). This award (entire period) has resulted in the publication of 9 full papers, two reviews, 7 abstracts, and we have two additional papers to be submitted within the next month. Thus, we have made substantial progress in defining initial components of a broader antiestrogen signaling network that is directly associated with acquired antiestrogen resistance.

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## Table of Contents

## INTRODUCTION

Antiestrogens have been successfully used in the management of breast cancer since the first clinical trial of Tamoxifen (TAM) in 1971 (1). TAM produces a significant increase in both overall and recurrence-free survival but resistance almost inevitably arises in most patients (2,3). We hypothesize that one form of acquired antiestrogen resistance reflects the altered expression of what were previously estrogen-regulated genes. We further hypothesize that only a subset of all estrogen (E2)-regulated genes, those comprising a specific gene network, is responsible for the resistance phenotype. Since TAM (triphenylethylene) and ICI 182,780 (steroidal) induce different ER conformations, we also hypothesize that the consequent patterns of gene regulation will be different and dictate the presence/absence of crossresistance among antiestrogens.

To address these hypotheses, we have generated novel E2-independent and antiestrogen resistant variants of the E2-dependent, MCF-7 human breast cancer cell line (MCF7/MIII, MCF7/LCC1, MCF7/LCC2, MCF-7/LCC9) - recently reviewed in (4). We also have assembled a panel of additional resistant cells from within this institution and from other investigators. These include additional antiestrogen resistant MCF-7 variants (LY2, R27, R3, MCF-7RR), all of which express ER, and the ER-negative ZR-75-1 (ZR75/LCC3, ZR-75-9a1) and T47D (T47Dco) variants. Other resistance models are currently being obtained from other laboratories or being generated by selection *in vivo* selection against TAM in athymic nude rats (rats and humans perceive TAM as a partial agonist, mice perceive TAM as a pure agonist).

This is an Idea Award to study the genes and patterns of genes expressed in acquired antiestrogen resistance in cell culture models. The PI will apply new, state-of-the-art technologies to identify key endocrine-regulated molecular pathways to apoptosis/proliferation. By identifying key components of these pathways, we may be able to predict response to first-line and crossover antiestrogenic therapies, and/or provide novel therapeutic strategies for antiestrogen resistant tumors.

**Antiestrogen Resistance.** Most breast tumors that initially respond to TAM recur and require other endocrine or cytotoxic therapies (3). Despite over 10 million patient years of experience with TAM, the precise mechanisms that confer acquired resistance are unknown (4). Absence of ER expression is clearly important for *de novo* resistance (4). ER expression is <u>not</u> lost in most breast tumors that acquire antiestrogen resistance (5). Currently, there is little compelling evidence that expression of ER splice variants and mutant ER contribute significantly to antiestrogen resistance in patients (4). While the importance of wild type ERα is established as a mediator/predictor of antiestrogen responsiveness, that of ERβ remains unclear. ERα may be the predominant species in most ER+ breast tumors (6,7), and is associated with a better prognosis (8). ERβ is associated with a poorer prognosis, absence of PgR, and lymph node involvement but a major role for this receptor in antiestrogen resistance seems unlikely (9,10). Some studies report activities independent of ER function, which may initiate events that are necessary but not sufficient for antiestrogen-induced effects (4). Our research team has recently reviewed in detail the potential mechanisms of antiestrogen resistance (9) and the role of antiestrogen regulated signaling to apoptosis (submitted).

## BODY OF REPORT

Our purpose was to evaluate a series of antiestrogen responsive and resistant breast cancer cell lines for their patterns of gene expression. We explored these data using state-of-the-art pattern analysis and statistically-based methods that apply both statistical and information theory. We also applied the more computationally simplistic methods used by others in the field.

## Specific Aims (unchanged)

**Specific Aim 1:** use gene microarrays to identify differentially expressed genes in a panel of breast cancer cell lines.

**Specific Aim 2:** explore the data from Aim 1 to identify those differentially expressed gene clusters most closely associated with acquired antiestrogen resistance and test further novel algorithms for the analysis of gene expression microarray data.

**Specific Aim 3:** begin to assess the likely functional relevance of representative members of these clusters and study their expression in human breast cancer biopsies.

**Long term aims:** establish a pattern(s) of gene clusters that can predict antiestrogen responses in patients. This could lead to a more effective identification of candidates for specific antiestrogen therapies and identify those patients least likely to respond and who may benefit from an early initiation of cytotoxic chemotherapy.

### KEY RESEARCH ACCOMPLISHMENTS

We include a full list of publications arising from this study (below) that demonstrate our successful completion of the proposed aims. The appendix includes reprints of key publications not included in prior annual reports.

**TASK 1:** *Use gene microarrays to identify differentially expressed genes in a panel of breast cancer cell lines.*

We have essentially completed this aim. Given the limited resources available during the no-cost extension, we chose not to do additional studies with the Affymetrix arrays but to focus on functional analyses of candidate genes (Task 3) and complete several publications. Thus, no new data have been generated since the previous annual report.

**TASK 2:** *Explore the data from Aim 1 to identify those differentially expressed gene clusters most closely associated with acquired antiestrogen resistance.*

We have also completed this Task, using data from both the Clontech and Research Genetics platforms. In-depth analyses/mining were done in the Mathworks MATLAB™ environment with

established and developing algorithms (see previous annual reports and reprints). Thus, no new data have been generated since the previous annual report.

**TASK 3:** *Begin to assess the likely functional relevance of representative members of these clusters and study their expression in human breast cancer biopsies.*

As previously described in earlier annual reports, to maintain focus within this application we have limited our studies to NFkB and IRF-1. We have now completed and published the data alluded to in last year's annual report where we implicated IRF-1 in antiestrogen-mediated signaling to apoptosis (see Bouker *et al.* Cancer Res, 2004). A copy of this paper is now included in this final report. We have also continued to made good progress in our studies on NFkB. We have now extended these observations to show that a small molecule inhibitor of NFkB (parthenolide) sensitizes resistant cells to the proapoptotic effects of the steroidal antiestrogen Faslodex (ICI 182,780). These data have been accepted, pending minor revisions (in progress), for publication in the journal *Molecular Cancer Therapy.* We include a copy of the submitted manuscript in the appendix to this report. We have also submitted a review of endocrine resistance and apoptosis that discusses some of our data in more detail while reviewing other related studies in the field.

## Key Research Accomplishments (bulleted)

- Completed and published manuscript describing the role of IRF-1 in ICI 182,780 mediated cell signaling and antiestrogen resistance.
- Completed and submitted manuscript (accepted pending minor revisions) describing the ability of the NFkB inhibitor to sensitize resistant cells to an antiestrogen.
- We have two additional papers to be submitted within the next month.
- The work supported through the entire period of this award resulted in the publication of 9 full papers, two reviews, and 7 abstracts

We believe that we have successfully addressed the goals of this award in an effective and productive manner. The reportable outcomes (publications) from this award are listed below.

## Reportable Outcomes

Reportable outcomes are presented as manuscripts and abstracts.

## Manuscripts and Abstracts (for entire grant period)

1. Ellis, M., Davis, N., Coop, A., Liu, M., Schumaker, L., Lee, R.Y., Srikanchana, R., Russell, C., Singh, B., Miller, W.R., Stearns, V., Pennanen, M., Tsangaris, T., Gallagher, A., Liu, A., Zwart, A., Hayes, D.F., Lippman, M.E., Wang, Y. & Clarke, R. "Development and validation of a method for using breast core needle biopsies for gene expression microarray analyses." *Clin Cancer Res,* 8: 1155-1166, 2002.

2. Wang, Y., Lu, J., Lee, R. & Clarke, R. "Iterative normalization of cDNA microarray data." *IEEE Trans Inf Techol Biomed,* 6: 29-37, 2002.

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4. Welch, J.N. & Clarke, R. "ErbB-2 expression and drug resistance in cancer." *Signal,* 3: 4-9, 2002.

5. Pratt, M.A.C., Bishop, T.E., White, D., Yasvinski, G., Ménard, M., Niu, M.Y. & Clarke, R. "Estrogen withdrawal-induced NF-κB and Bcl-3 expression in breast cancer cells: roles in growth and hormone independence." *Mol Cell Biol,* 23: 6887-6900, 2003.

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8. Han, F., Miksicek, R., Clarke, R. & Conrad, S.E. "Expression of an estrogen receptor variant lacking exon 3 in derivatives of MCF-7 cells with acquired estrogen independence or Tamoxifen resistance." *J Mol Endocrinol* 32: 935-945, 2004.

9. Bouker, K.B., Skaar, T.C., Fernandez, D.R., O'Brien, K.A., Riggins, R.B., Honghua, C. & Clarke, R. "Interferon regulatory factor-1 mediates the proapoptotic but not cell cycle arrest effects of the steroidal antiestrogen ICI 182,780 (Faslodex, Fulvestrant)." *Cancer Res,* 64:4030-4039, 2004.

10. Riggins, R.B., Nehra, R., Zwart, A., Agarwal, P. & Clarke, R. "The NFκB inhibitor parthenolide restores ICI 182,780 (Faslodex; Fulvestrant)-induced apoptosis in antiestrogen resistant breast cancer cells." *Mol Cancer Ther* (accepted pending minor revisions).

11. Riggins, R.B., Bouton, A.H., Liu, M.C. & Clarke, R. "Antiestrogens, aromatase inhibitors, and apoptosis in breast cancer." *Vit Horm* (submitted – review of this and related work).

Reprints of papers #1-#7 were included in previous annual reports; reprints of #8-#9, and copies of the submitted manuscripts #10-#11 are included with this final report.

Two other manuscripts are in the final stages of preparation for submission:

12. Zhu, Y., Singh, B., Hewitt, S., Liu, A., Gomez, B., Wang, A. & Clarke, R. "Expression patterns among proteins associated with endocrine responsiveness in breast cancer: interferon regulatory factor-1, human X-box binding protein-1, nuclear factor kappa B, nucleophosmin, estrogen receptor-alpha, and progesterone receptor."

13. Bouker, K.B., Skaar, T.C., Hamburger, D.S., Riggins, R.B., Fernandez, D.R., Zwart, A., Wang, A. & Clarke, R. "Tumor suppressor activities of interferon regulatory factor-1 in human breast cancer cells."

## Abstracts

1. Welch, J.N., Chrysogelos, S. & Clarke, R. "Expression and function of the epidermal growth factor receptor in breast cancer cells exposed to chemotherapy." *Proc Am Assoc Cancer Res* 42: 938, 2001.

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4. Zwart, A., Lee, R.Y., Zhang, J., Wang, J., Wang, Y. & Clarke, R. "mRNA profiles from MCF-7 variants are used to predict antiestrogen resistance/responsive phenotypes." *Proc 85th Annual Meeting Endocrine Society* 146, 2003.

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## Conclusions

Support provided through this DOD IDEA award has enabled us to make excellent progress in our studies on the molecular characterization of antiestrogen resistance. This progress is evident in our productivity as measured by publications (9 papers, 2 reviews, 7 abstracts, and 2 manuscripts in the final stages of preparation). The goals outlined in the original application have been met mostly as initially envisioned and the studies have generated preliminary data to support further funding applications and publications. For example, we expect to generate at least one other manuscript on the classifiers (not yet prepared). Our new data with Faslodex and parthenolide (see submitted manuscript in appendix) raise the possibility that clinical trials with these drugs may produce significant improvements in patient responses. The studies with IRF-1 and its dominant negative have identified a new signaling pathway through which some breast cancer regulated apoptosis (11). The role of NFkB in endocrine responsiveness also has been more clearly identified (12). Most recently, we have been able to detect expression of the protein products of all of the genes implicated and studied so far (NFkB, IRF-1, nucleophosmin and X-Box binding protein-1) in breast cancer biopsies (in preparation). Thus, our *in vitro* and human specimen data with NFkB, IRF-1 and other genes continue to contribute to our construction of a broader signaling network associated with acquired antiestrogen resistance (9). This exciting and productive study could not have been done without the support of this award.

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## Appendices.....

1. Han, F., Miksicek, R., Clarke, R. & Conrad, S.E. "Expression of an estrogen receptor variant lacking exon 3 in derivatives of MCF-7 cells with acquired estrogen independence or Tamoxifen resistance." *J Mol Endocrinol* 32: 935-945, 2004.
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# Expression of an estrogen receptor variant lacking exon 3 in derivatives of MCF-7 cells with acquired estrogen independence or tamoxifen resistance

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## Abstract

The estrogen receptor (ER) plays important roles in the development and progression of breast cancer, and is a major target for tumor therapy. In this study, we investigated ER function in two derivatives of MCF-7 cells that were selected for their ability to proliferate in the absence of estrogen or in the presence of the antiestrogen, tamoxifen. Reporter gene assays indicated decreased ER activity in both cells lines, although the activity remaining retained responsiveness to both estrogen and tamoxifen. The decreased ER activity correlated with expression of a 61 kDa variant ER protein, and sequencing of RT-PCR products indicated that this variant was the product of an exon 3 deletion (ERΔE3). To study its effects on cell proliferation, ERΔE3 cDNA was stably transfected into both the MCF-7 cell line and its estrogen-independent/tamoxifen-sensitive derivative MCF-7/LCC1 (LCC1), and the phenotypes of transfectants were examined. Expression of ERΔE3 was not sustainable in MCF-7 cells, but was maintained for at least 17 passages in LCC1 cells. These results are in agreement with previous reports that ERΔE3 inhibits wild-type ER activity and negatively regulates proliferation of MCF-7 cells. They further suggest that the alteration that leads to estrogen independence in LCC1 cells allows for sustained expression of ERΔE3, and that additional changes are required to confer tamoxifen resistance to these cells.

*Journal of Molecular Endocrinology* (2004) **32,** 935–945

## Introduction

Numerous studies have demonstrated the critical role that estrogen plays in the initiation and progression of breast tumors (Soule & McGrath 1980, Henderson *et al.* 1988). Based on these findings, endocrine therapies have been developed and used for the treatment of breast cancer patients. Tamoxifen is the most commonly used endocrine therapy in hormone-responsive breast cancer, especially as adjuvant therapy after removal of the primary tumor (Jordan 2000). Worldwide clinical trials indicate that the 5- and 10-year mortality rates of breast cancer patients can be reduced 20–25% by tamoxifen treatment (Early Breast Cancer Trialists' Collaborative Group 1992). Nevertheless, many tumors that initially respond to tamoxifen therapy eventually become resistant, and the development of such resistance in advanced breast cancer is a common cause for treatment failure. Recent reports indicate that aromatase inhibitors, which block production of estrogens, may be as or more effective than tamoxifen for the treatment of primary breast cancer (Milla-Santos *et al.* 2003). However, breast cancer cells can become estrogen independent, and the development of aromatase-resistant tumors might therefore occur. Understanding the molecular changes that take place during the evolution of resistance to endocrine therapies could provide strategies for preventing or treating such tumors. Several mechanisms might account for the development of resistance, including loss of estrogen receptor (ER), mutation of ER, alteration

of ER cofactor(s), abnormal metabolism, and alterations in growth factor pathways (Osborne *et al.* 1995, Jordan 1998, Clarke *et al.* 2001*a*). ER mutations have been observed in some cell lines selected *in vitro* for resistance to antiestrogens, as well as in tumor samples (McGuire *et al.* 1992, Wolf & Jordan 1994). However, many estrogen-independent and/or antiestrogen-resistant cell lines still express wild-type (wt) ER at significant levels (Jiang *et al.* 1992), suggesting that other mechanisms also exist.

Cell lines selected for the ability to grow in the absence of estrogen or in the presence of antiestrogen have been used as laboratory models of acquired endocrine resistance. MCF-7 cells have been widely used as a model of estrogen-dependent and antiestrogen-sensitive breast cancer. Both estrogen-independent and tamoxifen-resistant derivatives of MCF-7 have been selected using a protocol that mimics disease progression in patients (Leonessa *et al.* 1992, Brunner *et al.* 1993*a*,*b*, Thompson *et al.* 1993). MCF-7 cells were inoculated into ovariectomized athymic nude mice to select for estrogen-independent growth *in vivo* (Clarke *et al.* 1989). LCC1 cells were isolated from a rare tumor that developed, and were characterized as estrogen independent but tamoxifen sensitive (Brunner *et al.* 1993*a*). LCC1 cells were further selected *in vitro* for growth in the presence of 4-hydroxytamoxifen (4-OH TAM), giving rise to the MCF-7/LCC2 (LCC2) cell line, which is resistant to tamoxifen but still sensitive to steroidal antiestrogens such as ICI 182,780 (Brunner *et al.* 1993*b*). The phenotype of LCC2 cells is similar to that seen in many breast cancer patients who develop tamoxifen resistance during endocrine therapy (Howell *et al.* 1995).

The current study was aimed at identifying biochemical changes that occurred during the evolution of LCC1 and LCC2 cells. Estrogen response element (ERE) reporter gene assays demonstrated that although ER activity in LCC1 and LCC2 cells was regulated by 17-β estradiol and 4-OH TAM, the overall level of expression of ERE reporter genes was significantly lower in LCC1 than in MCF-7 cells, and even lower in LCC2 cells. Western blot analyses revealed a 61 kDa ER variant expressed in LCC1 and at higher levels in LCC2 cells, and sequencing of RT-PCR products identified this variant as one containing a deletion of exon 3. The ERα gene has eight coding exons, which generate a 6·2 kb wtER mRNA. Aberrant mRNA splicing can produce ER variant mRNAs with various exon deletions (Wang & Miksicek 1991, Koehorst *et al.* 1993, Miksicek *et al.* 1993, Sluyser 1994, Gotteland *et al.* 1995, Pfeffer *et al.* 1995, Hopp & Fuqua 1998, Murphy *et al.* 1998, Fasco *et al.* 2000). Variant ER mRNAs have been detected in normal breast tissue, breast cancer cell lines, and clinical tumor samples using RT-PCR. They are observed in the majority of ER-positive tumors and tumor cell lines, and attempts have been made to correlate changes in their expression with clinical features. In one study of breast tumors, expression of a specific ER variant correlated with antiestrogen resistance (Daffada *et al.* 1995). However, examination of *in vitro*-derived antiestrogen-resistant cell lines failed to observe any consistent changes in ER variant mRNA expression as a function of tamoxifen, ICI 164,384, or ICI 182,780 resistance (Madsen *et al.* 1997). In addition, although variant ER mRNAs are readily detected, the level of variant protein is usually extremely low or undetectable, making the physiological role of the RNAs questionable.

Among the characterized ER variants, one of the most commonly seen is the exon 3 deletion (ERΔE3). Exon 3 encodes the second zinc finger of the DNA binding domain. The ERΔE3 protein does not bind to EREs and does not activate EREs in transient transfection assays, but can dimerize with both itself and wtER. When ERΔE3 was mixed with wtER in a ratio of 1:1, ERE activity was inhibited by 30%, suggesting that ERΔE3 functions as a dominant negative variant (Wang & Miksicek 1991). Consistent with this finding, ectopic expression of ERΔE3 in MCF-7 cells has been reported to inhibit anchorage independent growth and to suppress their invasiveness (Erenburg *et al.* 1997). However, ERΔE3 has also been reported to be a potent activator of some promoters containing AP1 sites (Bollig & Miksicek 2000), and it is therefore possible that it might promote proliferation under some circumstances.

The discovery that ERΔE3 protein is present in LCC1 and LCC2 cells suggested that they might be resistant to the inhibitory effects of this ER variant. Stable transfection experiments demonstrated that this was the case. Expression of ERΔE3 was strongly selected against in MCF-7 cells, but not in LCC1 cells. This is consistent with a model in which a change has occurred in LCC1 cells that

makes them partially independent of ER function. However, this independence is not complete, since both LCC1 cells and derivatives expressing high levels of ERΔE3 retain sensitivity to tamoxifen.

## Materials and methods

### Cell culture

MCF-7, LCC1 and LCC2 cells were maintained in improved modified Eagle's medium (IMEM) (Biofluids, Inc., Rockville, MD, USA) supplemented with 5% fetal bovine serum (Hyclone Logan, Utah, USA), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Invitrogen Life Technologies). To study the effects of estrogen and antiestrogens, cells were grown in IMEM without phenol red supplemented with 5% charcoal-stripped fetal bovine serum (CSS) (Hyclone).

### Reagents and plasmids

17β-Estradiol and 4-OH TAM were purchased from Sigma (Sigma Aldrich, St Louis, MO, USA). ICI 182,780 (ICI) was from AstraZeneca. Lipofectin was purchased from Life Technologies. The ERΔE3 and wtER expression vectors (pCDNA3-ERΔE3 and pCDNA3-wtERα) were constructed by insertion of the ERΔE3 and wtERα cDNAs into pCDNA3 (Invitrogen). pβgal-Basic was purchased from Clontech Laboratories (Palo Alto, CA, USA). The ERE-luciferase reporter construct, ERE2-tk109-luc, was obtained from Dr Gehm at Northwestern University Medical School (Gehm *et al.* 1997).

### Primers and PCR conditions

The primers used for RT-PCR were 5′-CTGCCA AGGAGACTCGCTAC-3′ (upstream) and 5′-AAG GCACTGACCATCTGGTC-3′ (downstream). The primers used for genomic PCR were 5′-CGCTCG AGTGGGGTGCAACGTAGTAAGA-3′ (upstream) and 5′-GCGAATTCCAATGGGTAGAGCCAG-3′ (downstream). For reverse transcription reactions, 5 μg total RNA and 10 pmol of the downstream primer were incubated for 10 min at 65 °C in deionized $H_2O$, and then in a total of 20 μl reverse transcription mixture (50 mM Tris–HCl, pH 8·3, 40 mM KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 50 μM dNTPs, 200 units MMLV-Reverse Transcriptase (Invitrogen Life Technologies) for 1 h at 37 °C. For PCRs, 4 μl of the reverse transcription mixture or 1 μg genomic DNA were amplified in a final volume of 100 μl containing 250 nM of each primer, 200 μM dNTPs, 1·5 mM $MgCl_2$, and 2·5 units TAQ DNA polymerase (Invitrogen Life Technologies). Each RT-PCR consisted of 35 cycles (60 s at 62 °C, 60 s at 74 °C and 30 s at 94 °C). Each genomic PCR consisted of 40 cycles (60 s at 58 °C, 60 s at 74 °C and 60 s at 94 °C). PCR products were visualized on agarose gels stained with ethidium bromide. The DNA fragments of interest were then recovered and purified using a GenElute Agarose Spin Column (Sigma), and sequenced at the Michigan State University Genomic Technology Support Facility.

### Western blotting

To prepare cell extracts, monolayers (80% confluent) were washed twice in ice-cold PBS and collected by scraping and centrifugation. Cell pellets were resuspended in ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, pH 7·5, 1 mM EDTA, 2·5 mM EGTA, 0·1% Tween-20, 10% glycerol, 1 mM DTT, 0·1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 10 mM β-glycerophosphate and 1 mM NaF) and lysed by sonication on ice (duty cycle 80%, output 6, 10 s) using a Sonifier 450 (Branson Ultrasonics Corporation, Danbury, CT, USA). The cellular debris was removed by centrifugation (12 000 ***g***, 2 min, 4 °C). The total protein concentration in the cell lysate was measured using a Bradford assay (Bio-Rad Laboratories, Inc. Hercules, CA, USA). Twenty micrograms total protein from each sample were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (NEN Life Science Products, Perkin Elmer, Boston, USA) in transfer buffer (25 mM Tris, 192 mM glycine and 15% methanol) using the XCELL gel transfer system (Invitrogen Life Technologies). Western analysis of ER protein levels utilized a mouse monoclonal antibody (Mab-17) that was raised against recombinant ERα protein, and which recognizes an amino-terminal epitope present in both wtER and ERΔE3. Membranes were blocked in 5% dry milk in PBST (PBS+0·1% Tween 20) and incubated with a 1:2 dilution of the Mab-17 in PBS at 4 °C overnight. The antibody was removed and the membrane was washed with PBST three times for 10 min each. A peroxidase-labeled

secondary antibody (American Qualex, San Clemente, CA, USA) was diluted 1:2000 in 5% dry milk in PBST and incubated with the membrane for 1 h at room temperature. Blots were reprobed with an antibody to β-actin as a loading control. Proteins were then visualized using Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

### Transient transfection and luciferase assays

Cells were plated in IMEM containing 5% FBS at $4 \times 10^5$ cells per 60 mm tissue culture plate, and incubated overnight. Each plate was then incubated with 5 μg ERE2-tk109-luc and 1 μg pβgal-Basic using Lipofectin (Invitrogen Life Technologies) as the transfection agent. Lipofectin/DNA complexes were formed in phenol red-free, serum-free IMEM, and left on plates for 6 h. The transfection medium was then removed, cells were washed twice in PBS and then incubated in IMEM supplemented with 5% CSS for 48 h to deprive them of estrogen. Fresh medium containing 5% CSS with or without estrogen ($10^{-9}$ M), 4-OH TAM ($10^{-6}$ M), or ICI ($10^{-7}$ M) was then added for 8 h. Treatments were added as stock solutions in absolute ethanol, and ethanol was added to control media to the same final concentration in all plates. After the 8 h treatment, cells were washed twice with PBS and harvested by scraping and centrifugation. Cell pellets were resuspended in 200 μl reporter lysis buffer (Promega Corp., Madison, WI, USA) and lysed by freezing and thawing. Protein concentrations in cell extracts were determined using the Modified DC assay (Bio-Rad). Aliquots of each lysate were assayed for β-galactosidase (Clontech) and luciferase (Promega) activities on a Turner TD-20e luminometer using protocols suggested by the manufacturer. The luciferase activity was normalized to the β-galactosidase activity in the same extract, and is shown as the mean ± S.D. of three independent transfections.

### Stable transfections

To obtain stable cell lines, MCF-7 and LCC1 cells were transfected with pCDNA3-ERΔ3 or pCDNA3-wtERα using the Lipofectin reagent, and selected in medium containing 400 μg/ml G418 (Geneticin, Amersham BioSciences, Piscatawy, NJ, USA). Individual G418 resistant colonies were isolated, and expanded into cell lines that were subsequently maintained in media containing 40 μg/ml G418. Passage one was defined as the point at which each transfected cell line was passed to a single 10 cm plate. Clonal cell lines that expressed ERΔE3 or wtER were identified by Western blotting.

### Colony formation in soft agar

Cells were plated in phenol red-free IMEM containing 5% CSS for two days, then harvested by trypsinization. Cells were suspended in IMEM containing 5% CSS, 0·3% Nobel agar (Difco Laboratories, BDDiagnostic Systems, Sparks, MD, USA) in the presence or absence of estrogen or 4-OH TAM. They were then plated at a density of $5 \times 10^4$ cells/well in 6-well dishes on top of a layer of IMEM containing 5% CSS plus or minus estrogen or 4-OH TAM and 0·6% agar. The plates were incubated for 21 days, with feeding every 5 days. Colonies were then stained with 0·1% neutral red and analyzed by microscopy. Colonies larger than 60 μm (more than 50 cells) were counted, and six independent wells per treatment were averaged. The results were expressed as means ± S.D.

## Results

### ER activity in MCF-7, LCC1 and LCC2 cells

To investigate whether ER activity or regulation was altered in LCC1 or LCC2 cells, expression and regulation of an ERE-Luc reporter gene in these cell lines were compared with MCF-7 cells. An ERE-luciferase plasmid construct (ERE2-tk109-luc) was co-transfected into cells along with a control β-galactosidase construct (pβgal-Basic) as described in Materials and methods. After transfection, cells were treated with vehicle, estrogen, 4-OH TAM, or ICI for 8 h, harvested and analyzed for luciferase and β-galactosidase activities as described in Materials and methods. As shown in Fig. 1, the adjusted luciferase activity was low in all three cell lines in CSS, although it was significantly higher in MCF-7 than in LCC1 ($P$=0·0003) or LCC2 ($P$<0·0001) cells. Estrogen induced luciferase expression, and this induction was reversed by both ICI and 4-OH TAM in all of the cell lines. This indicates that both estrogen and antiestrogens regulate ER activity in LCC1 and LCC2 cells. However, the magnitude of luciferase induction by

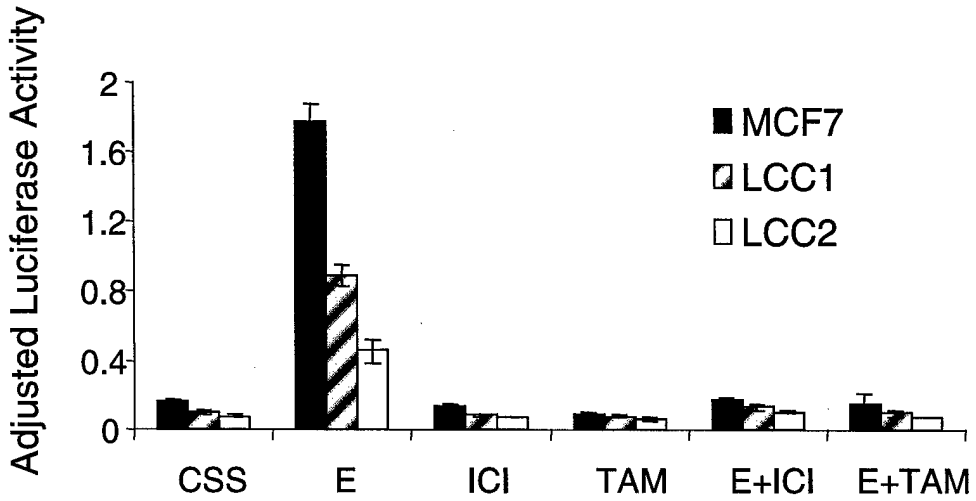


**Figure 1** ER activity in MCF-7, LCC1 and LCC2 cells. ER function was assessed by co-transfection of ERE-luciferase and β-gal reporter genes into MCF-7, LCC1, and LCC2 cells as described in Materials and methods. Transfected cells were treated with estrogen (E; $10^{-9}$ M), 4-OH TAM (TAM; $10^{-6}$ M), or ICI 182,780 (ICI; $10^{-7}$ M) for 8 h, then harvested and assayed for luciferase and β-galactosidase activities. The adjusted luciferase activity was the luciferase activity divided by the β-galactosidase activity in the same extract. The data presented represent the means±S.D from three independent transfections.

estrogen was significantly lower in LCC2 than in either MCF-7 ($P$=0·008) or LCC1 ($P$=0·0055) cells. Together with the lower basal ER activity in LCC1 and LCC2 cells, these results suggested that ER function might be altered in LCC1 and/or LCC2 cells, despite the fact that ER levels and binding affinities in these cell lines were previously shown to be comparable to MCF-7 cells by ligand binding assays (Brunner *et al.* 1993*b*).

### Identification of ERΔE3 protein in LCC1 and LCC2 cells

To investigate the cause of the altered ER activity in LCC1 and LCC2 cells, Western blot analyses of ERα protein were carried out. MCF-7, LCC1 and LCC2 cells were incubated in IMEM/CSS for 2 days, and then treated with estrogen, 4-OH TAM, or ICI. After 2 days of treatment, cells were harvested and lysates were analyzed by Western blotting using a monoclonal antibody that recognizes an N-terminal epitope of wtER. ER expression levels were similar in the three cell lines (Fig. 2). Interestingly, an ER variant of approximately 61 kDa was detected at high levels in LCC2, and to a lesser extent in LCC1, cells. In this experiment, the level of the 61 kDa ER variant was comparable to that of the 66 kDa wtER in LCC2 cells. In addition, expression of the 61 kDa ER variant was regulated by

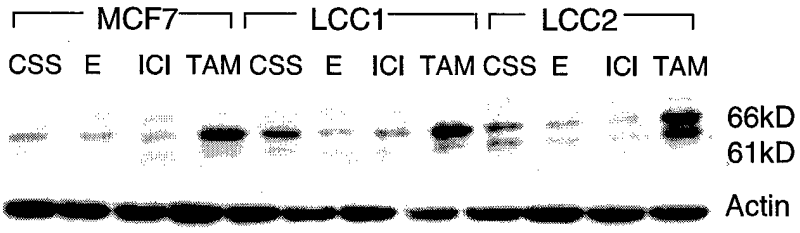


**Figure 2** Analysis of ER protein expression in MCF-7, LCC1 and LCC2 cells. MCF-7, LCC1, and LCC2 cells were treated with estrogen (E), 4-OH TAM (TAM), or ICI 182,780 (ICI) for 48 h. Cell extracts were prepared and analyzed for ER expression by SDS-polyacrylamide gel electrophoresis and Western blotting as described in Materials and methods.

estrogen, 4-OH TAM and ICI in the same way as that of wtER.

To investigate the nature of the ER variant in LCC2 cells, it was molecularly characterized. Based on the size of the protein, it was suspected to arise from a deletion of exon 3. To test this hypothesis, a set of primers was designed to span exon 3, with the upstream primer located in exon 2 and the downstream in exon 4. Total RNA was isolated from MCF-7 and LCC2 cells, and RT-PCRs were performed as described in Materials and methods. Control PCRs were carried out in parallel with cDNA constructs containing either the wild-type or Δ3 ER coding sequences. As shown in Fig. 3a, RT-PCRs using MCF-7 cell RNA gave rise to a single band of the predicted 420 bp that co-migrated with the fragment amplified from the wtER cDNA. This fragment was also present in the RT-PCR products from LCC2 cells; however, the LCC2 cell products also included a 320 bp fragment that co-migrated with the fragment amplified from the ERΔE3 cDNA. To confirm that the 320 bp fragment amplified from LCC2 cells indeed represented an exon 3 deletion, it was purified and sequenced. The sequencing results established that LCC2 cells contain significant amounts of an mRNA species containing a precise deletion of exon 3 (Fig. 3b), which is likely to account for the 61 kDa ER variant protein detected by Western blotting.

One potential mechanism for the generation of the ERΔE3 mRNA found in LCC2 cells would be a splice site mutation in one allele of the genomic DNA. To test for such a mutation, genomic PCR was carried out in MCF-7 and LCC2 cells using a primer set with the first strand primer located in intron 2 and the second in intron 3. The resulting 313 bp fragment containing exon 3 plus flanking intron sequences was then purified and sequenced. No genomic mutations were observed within 122 bp upstream or 77 bp downstream of exon 3 in either MCF-7 or LCC2 cells (data not shown), a region that typically includes all essential splicing signals including the branch point adenosine.

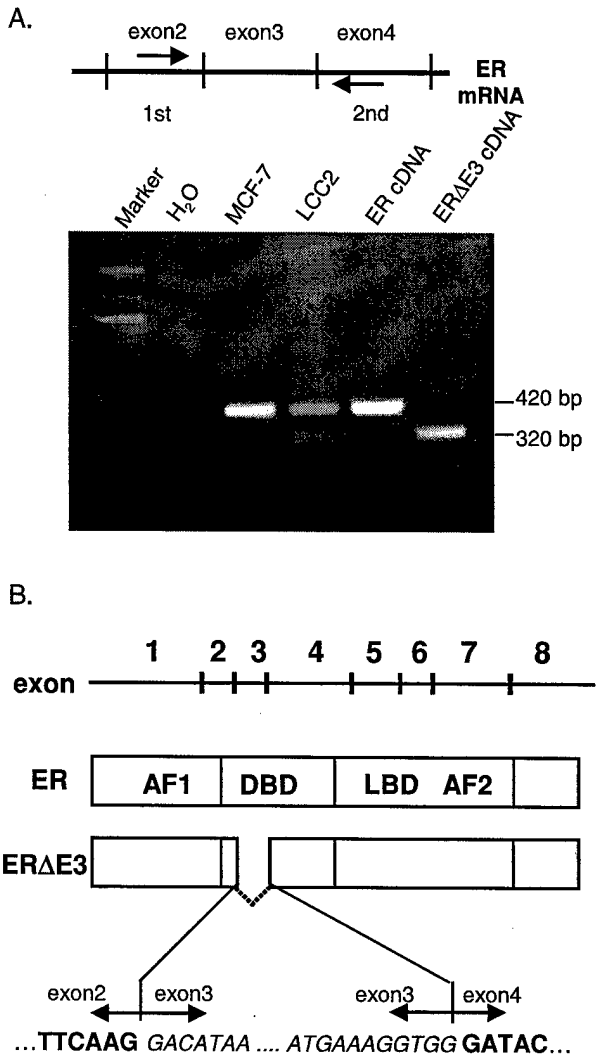


**Figure 3** Characterization of ER mRNAs expressed in LCC2 cells. (A) Total RNA was isolated from MCF-7 and LCC2 cells as described in Materials and methods. RT-PCRs were carried out using the indicated primers. PCRs with both wild-type ER and ERΔE3 cDNAs were included as controls. Final PCR products were separated on agarose gels and stained with ethidium bromide. (B) The 420 bp and 320 bp PCR products from MCF-7 and LCC2 cells were purified and sequenced, and the results are illustrated diagrammatically. The sequences found in both the 420 and 320 bp products are shown in bold letters, and those found only in the 420 bp fragment are shown in italics. DBD, DNA binding domain; LBD, ligand binding domain.

## Ectopic expression of ERΔE3 in stably transfected MCF-7 and LCC1 cells

Previous research has indicated that ERΔE3 inhibits wtER function at EREs, and also inhibits both anchorage-independent growth and invasiveness of transfected MCF-7 cells. In addition, ERΔE3 mRNA levels were higher in normal mammary epithelial cells than in breast cancer cells, suggesting that it may be involved in limiting

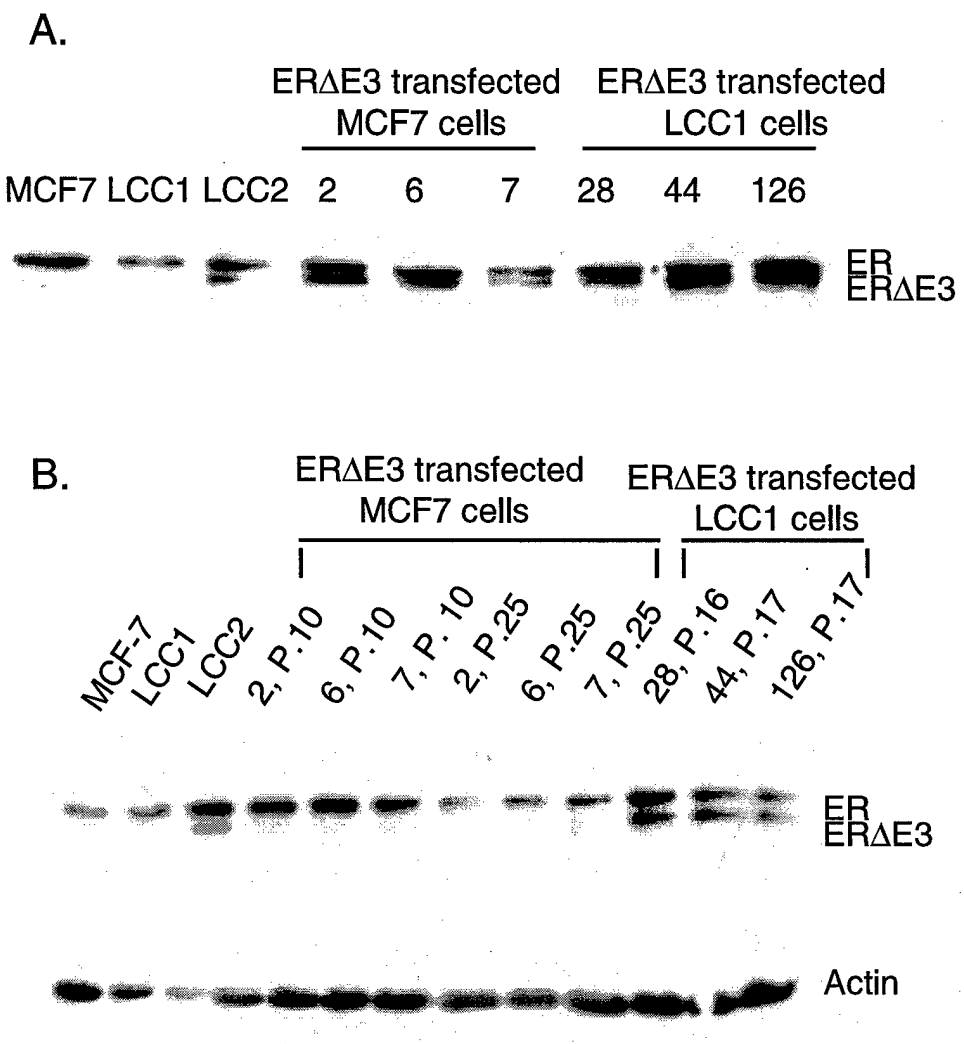


**Figure 4** ERΔE3 expression in stably transfected MCF-7 and LCC1 cells. MCF-7 and LCC1 cells were transfected with wtER and ERΔE3 cDNAs, and stable transfectants were selected as described in Materials and methods. (A) Western blot analysis of ER proteins expressed at the time of screening individual transfectants from each cell line. Lines 2, 6, and 7 represent three independent pCDNA3-ERΔE3 transfected MCF-7 cell clones. Lines 28, 44, and 126 represent three independent pCDNA3-ERΔE3 transfected LCC1 cell clones. Western blots of extracts from MCF-7, LCC1 and LCC2 cells are included as controls. (B) Western blot analysis of the ER protein present in later passages (P.10, P.25, P.16, P.17) of the cell lines shown in (A).

proliferation (Erenburg *et al.* 1997). Given these facts, the high levels of ERΔE3 protein in LCC2 cells, which have increased tumorigenic potential relative to MCF-7 cells, was surprising. To investigate whether expression of ERΔE3 has different consequences in MCF-7 cells and their estrogen-independent derivative, LCC1, stable transfections were carried out. MCF-7 and LCC1 cells were transfected with either pCDNA3-wtER or pCDNA3-ERΔE3, and G418$^r$ colonies were selected and expanded into clonal cell lines. Lines expressing ERΔE3 or wtER were identified by RT-PCR and confirmed by Western blotting. As shown in Fig. 4a, when cultures were analyzed soon (within 1–2 passages) after selection, three independent derivatives of both MCF-7 and LCC1

expressed the ERΔE3 gene at similar levels to the endogenous wild-type. However, later passages of the MCF-7-derived lines no longer expressed the 61 kDa ER variant (Fig. 4b), suggesting that it had been selected against. In contrast, the LCC1-derived lines maintained expression of the variant protein for at least 17 passages. These results indicate that a change occurred during the derivation of LCC1 cells that allows them to tolerate continued expression of ERΔE3.

### Anchorage-independent proliferation of LCC1 cells expressing ERΔE3

LCC2 cells consistently express higher levels of ERΔE3 protein than LCC1 cells and have also acquired tamoxifen resistance. To investigate whether expression of ectopic ERΔE3 affected the growth properties and/or tamoxifen sensitivity of LCC1 cells, the ability of control and transfected cell lines to form colonies in soft agar were compared. Cell lines examined included MCF-7, LCC1, LCC2, LCC1 cells stably expressing ERΔE3 or wtER, and LCC1 cells containing the pcDNA3 vector. Expression of ERΔE3 and wtER in the transfected cell lines at the start of these assays was confirmed by Western blotting of cell lysates (Fig. 5a). The ability of the different cell lines to form colonies in soft agar after a 3-week incubation in medium containing CSS, CSS+ estrogen, or CSS+4-OH TAM was evaluated by staining with neutral-red followed by microscopy (Fig. 5b) and direct counting of colonies (Fig. 5c).

As shown in Fig. 5, colony formation by MCF-7 cells was significantly higher in the presence of estrogen than in the absence of estrogen or in the

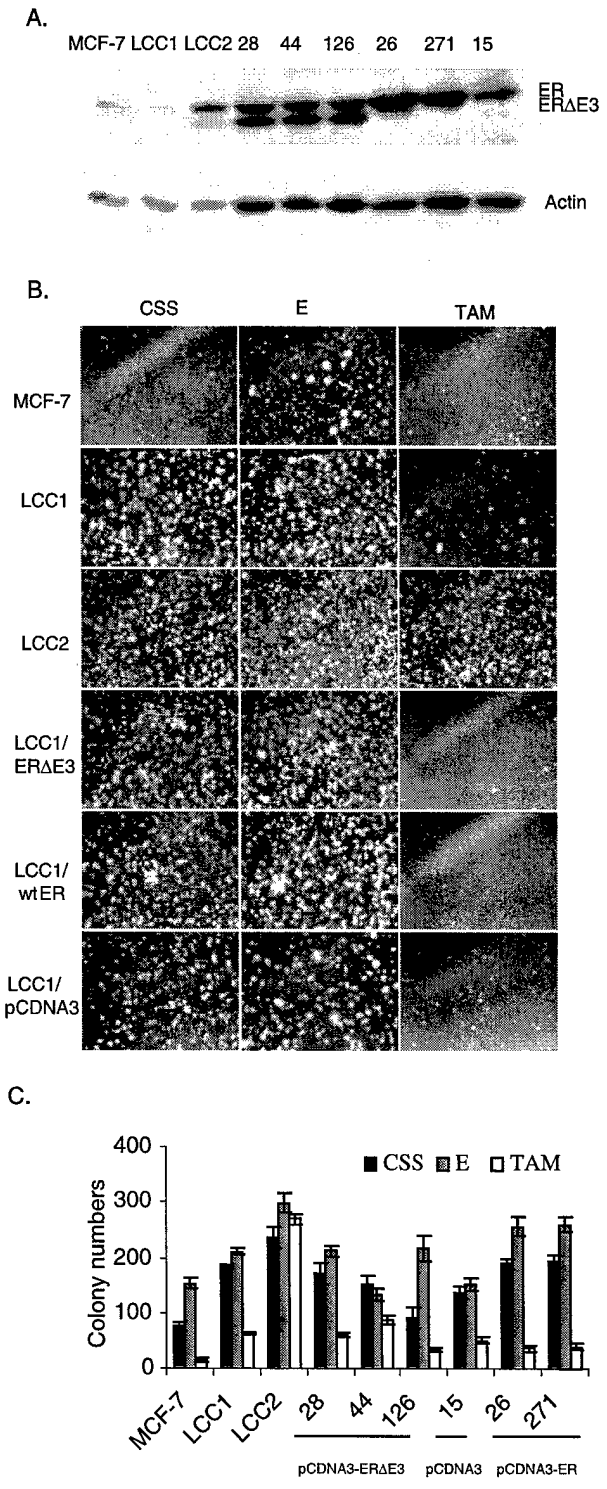


**Figure 5** Effects of ERΔE3 expression on the growth properties of LCC1 cells. (A) Expression of wtER and ERΔE3 in transfected cell lines was confirmed by Western blotting. Lines 28, 44, and 126 represent three independent pCDNA3-ERΔE3 transfected LCC1 cell clones. Lines 26 and 271 represent two independent pCDNA3-wtERα transfected LCC1 cell clones. Line 15 is a pCDNA3 transfected LCC1 cell clone. (B) Cells were plated in IMEM/CSS for 2 days and then grown in 0·3% soft agar with or without estrogen (E; $10^{-9}$ M) or 4-OH TAM (TAM; $10^{-6}$ M) for 21 days as described in Materials and methods. LCC1/ERΔE3 represents data from clone 28, which was similar to clone 44 and clone 126. LCC1/wtER represents data from clone 26, which was similar to clone 271. LCC1/pCDNA3 represents clone 15's data. (C) Colonies larger than 60 μm (more than 50 cells) were counted, and six independent wells per treatment per sample were averaged. The results are expressed as means±S.D

presence of 4-OH TAM ($P<0{\cdot}0001$). Colony formation by LCC1 cells was similar in the presence or absence of estrogen, but low in the presence of 4-OH TAM ($P<0{\cdot}0001$). In contrast, LCC2 cells formed colonies with similar efficiency under all conditions. Thus, the behavior of these cell lines in the soft agar assay clearly reflects their reported phenotypes (Clarke *et al.* 2001*a*). The phenotype of LCC1 cells stably expressing either wtERα or ERΔE3 was unchanged; in each case the number of colonies formed in the presence of 4-OH TAM was significantly less than in CSS or CSS plus estrogen ($P$ values ranged from 0·01 to $<0{\cdot}0001$) (Fig. 5c). Thus, expression of ERΔE3 does not inhibit LCC1 colony formation in the absence or presence of estrogen, nor does it confer tamoxifen resistance to these cells. Because ERΔE3 expression was not maintained in transfected MCF-7 cells (Fig. 4b), we were unable to directly examine the effects of ERΔE3 expression on colony formation in this cell line. However, a previous report indicated that expression of ERΔE3 inhibits the ability of MCF-7 cells to proliferate in soft agar in the presence of estrogen (Erenburg *et al.* 1997), and this is consistent with the data presented here.

## Discussion

In this study, we investigated ER expression and function in MCF-7-derived cell lines with acquired estrogen independence or tamoxifen resistance. We have shown that a specific ER variant, ERΔE3, is expressed at high levels in the estrogen-independent/tamoxifen-resistant LCC2 cell line, and to a lesser extent in estrogen-independent but tamoxifen-sensitive LCC1 cells. Although there have been numerous reports of ER variant mRNAs in breast cancer cells (Wang & Miksicek 1991, Koehorst *et al.* 1993, Miksicek *et al.* 1993, Sluyser 1994, Gotteland *et al.* 1995, Pfeffer *et al.* 1995, Hopp & Fuqua 1998, Murphy *et al.* 1998, Fasco *et al.* 2000), the existence of variant proteins has been more controversial, and this is one of the few cases where a significant amount of a naturally occurring ER variant is expressed at the protein level.

The identification of an ER variant that is expressed at high levels in breast cancer cell lines raises several interesting questions. One is the mechanism leading to expression of the variant protein, and the second is its effects on cellular phenotype. Previous studies have shown that several different variant ER mRNAs can co-exist in both normal and transformed tissues (Gotteland *et al.* 1995), suggesting that they generally do not result from simple mRNA splice site mutations. By sequencing PCR products from genomic DNA, we demonstrated that the presence of ERΔE3 mRNA in LCC2 cells is not a result of a simple splice site mutation (data not shown). The analysis carried out does not, however, rule out the complete loss of exon 3 and surrounding sequences from one allele of the ER gene in LCC2 cells. Early evidence suggested that exon skipping is one of the likely sources of ER variant mRNAs in breast cancer cells (Miksicek *et al.* 1993). The amount of ERΔE3 protein present in LCC1 and LCC2 cells is somewhat variable (compare Figs 2 and 5), which is consistent with this suggestion. However, the amount of ERΔ3 protein is consistently higher in LCC2 than LCC1 cells, indicating that expression of this variant is regulated.

In addition to investigating the source of the increased ERΔE3 in LCC2 cells, we have characterized its effects upon cellular phenotype, and found that the increased level of ERΔE3 from MCF-7 to LCC1 to LCC2 cells correlates with decreased activity of ERE reporter genes in these cell lines. Furthermore, since its expression is not maintained in stably transfected MCF-7 cells, ERΔE3 seems to inhibit the estrogen-dependent proliferation or survival of these cells. These findings are consistent with previous reports that ERΔE3 acts as a dominant negative mutant at EREs (Wang & Miksicek 1991, Erenburg *et al.* 1997, Bollig & Miksicek 2000). In contrast to the situation in MCF-7 cells, ERΔE3 expression can be maintained for many passages in LCC1 cells. The fact that ERΔE3 does not inhibit proliferation of LCC1 cells suggests that these cells no longer require the action of ER at ERE-regulated genes, and it seems likely that the same change(s) giving rise to this phenotype also confer estrogen independence. Such changes might include activation of genes that are normally induced by estrogen, increased expression or mutation of ER coregulators, or activation of growth factor pathways (Clarke *et al.* 2001*a*,*b*).

It is interesting to note that although LCC1 cells proliferate in the presence of what appears to be a dominant negative ER, they are still sensitive to tamoxifen, indicating that they are not completely

independent of ER function. There are several possible explanations for this result. One is that tamoxifen-bound ER may have unique effects upon some genes that regulate cell proliferation; for example it might suppress genes required for proliferation or activate genes that inhibit proliferation. Alternatively, ERΔE3 may activate genes that promote proliferation, either via interactions with transcription factors such as AP1, or via non-genomic mechanisms, and these activities may be inhibited by tamoxifen. Although our results indicate that such activities are not sufficient to promote proliferation, their inhibition might prevent it. Determining which, if either, of these explanations is correct would have important implications for understanding the mechanisms leading to tamoxifen resistance. Finally, although our data clearly demonstrate that expression of ERΔE3 is not sufficient to confer tamoxifen resistance to LCC1 cells, it is possible that increased ERΔE3 expression plays an indirect or contributory role in its development. The continuous presence of ERΔE3 may impose a selective pressure on LCC1 cells to become less dependent on ER function and, over time, may favor the emergence of tamoxifen-resistant progeny.

## Acknowledgements

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# Interferon Regulatory Factor-1 Mediates the Proapoptotic but Not Cell Cycle Arrest Effects of the Steroidal Antiestrogen ICI 182,780 (Faslodex, Fulvestrant)

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## ABSTRACT

**Antiestrogens induce both cytostasis (cell cycle arrest) and apoptosis, but the relationship between these end points and the signaling that regulates their induction are unclear. We have previously implicated the transcription factor and putative tumor suppressor IFN regulatory factor-1 (IRF-1) in acquired antiestrogen resistance (Gu *et al.*, Cancer Res, *62:* 3428–3437, 2002). We now show the functional significance of IRF-1 in affecting antiestrogen responsiveness in estrogen receptor-positive antiestrogen-sensitive models (MCF-7, T47D, and ZR-75-1), a model of acquired antiestrogen resistance (MCF7/LCC9; estrogen receptor positive), and a model of *de novo* antiestrogen resistance (MDA-MB-231; estrogen receptor negative). Basal IRF-1 mRNA expression is lower in MCF7/LCC9 cells when compared with MCF-7, T47D, and ZR-75-1 cells. IRF-1 transcriptional activity in MCF-7/LCC9 cells is 18-fold lower than that seen in the parental cells (MCF-7/LCC1) and is comparable with that in MDA-MB-231 cells. Although IRF-1 mRNA expression is induced by ICI 182,780 in sensitive cells, this regulation is lost in MCF-7/LCC9 and is absent in MDA-MB-231 cells. Loss of IRF-1 regulation appears specific to antiestrogen resistance—resistant cells induce IRF-1 mRNA in response to the cytotoxic drug doxorubicin. A dominant-negative IRF-1 eliminates the ICI 182,780-induced apoptotic response (reduced >4-fold) and reduces MCF-7 and T47D cell sensitivity to the antiproliferative effects of ICI 182,780. This effect is not mediated by changes in cell cycle distribution; rather, dominant-negative IRF-1 reduces ICI 182,780-induced apoptosis. These data identify a novel mechanism of antiestrogen resistance and implicate IRF-1 as a key component in signaling some ER-mediated effects on apoptosis/cell survival.**

## INTRODUCTION

For many women, antiestrogen therapy is the least toxic and most effective means to manage their hormone-dependent breast cancer. The most widely studied antiestrogen has been tamoxifen (TAM), which can increase both disease-free and overall survival in breast cancer patients, reduce the incidence of estrogen receptor-positive (ER+) disease in high-risk women, and reduce the rate of bone loss from osteoporosis (1, 2). Although first line antiestrogen therapy remains the standard of care for these patients (3–5), approximately one-third of all ER+ breast tumors exhibit *de novo* antiestrogen resistance, and most initially responsive tumors eventually acquire resistance (6).

The steroidal antiestrogen ICI 182,780 (Faslodex; Fulvestrant) has successfully completed clinical trials and exhibits considerable potential for more widespread clinical use (7). Most currently available antiestrogens show little or no significant activity in TAM-resistant disease, which is often treated with a second-line aromatase inhibitor. However, ICI 182,780 is clearly active in patients who have received TAM treatment and eventually recurred (8). Furthermore, two Phase III clinical trials in TAM-resistant patients have shown ICI 182,780 to be at least as effective as the potent aromatase inhibitor anastrazole (9, 10). Unlike most other antiestrogens, ICI 182,780 is a pure ER antagonist (11) that can induce degradation of ER protein (12) and inhibit receptor dimerization (13). Furthermore, ICI 182,780 is devoid of the uterotropic activity associated with the ability of TAM to increase the risk of developing endometrial cancers (8, 14).

Antiestrogen and estrogen responsiveness are complex phenotypes, and both genomic and nongenomic activities are functionally implicated (6, 15). In sensitive cells, antiestrogens are clearly cytostatic, inducing a $G_0$-$G_1$ cell cycle arrest *in vitro*. Clinically, the ability of antiestrogens to induce significant reductions in tumor size and increases in overall survival (1, 2) and to inhibit the development of ER+ tumors in the chemopreventive setting (16, 17) strongly suggest that these drugs also may be cytotoxic. Evidence implicates an induction of apoptotic cell death as the major mechanism through which antiestrogens might induce a cytotoxic effect (6). However, the relationship between growth arrest and apoptosis and how antiestrogens functionally affect cell signaling to regulate these two end points remains to be firmly established.

It is becoming apparent that antiestrogen resistance in ER+ tumors is unlikely to be driven by a single gene/signaling pathway. Thus, we have invoked a gene network hypothesis that confers diversity in estrogen/antiestrogen-initiated signaling (15, 18, 19). Ultimately, we envision multiple concurrent signals through this network of integrated and potentially interdependent pathways, some antiapoptotic and some proapoptotic, with cellular response reflecting the dominant signals. In antiestrogen-unresponsive cells, we hypothesize that the endocrine regulation and/or function of key components of this network is changed and that proapoptotic signals are no longer induced and antiapoptotic signals have become dominant. To begin identifying key genes that may make up such a network, we have applied both serial analysis of gene expression and gene expression microarray analyses to a series of antiestrogen-sensitive and -resistant cells. Among the key genes identified is the putative tumor suppressor gene IFN regulatory factor-1 (IRF-1; Ref. 19).

Although initially identified as an IFN-responsive gene, IRF-1 has shown activity as a tumor suppressor in several studies (20–22). For example, IRF-1 is deleted in some cancers (23, 24), and loss of IRF-1 significantly increases tumorigenicity in mouse models driven either by ras or loss of p53 (25). IRF-1 can signal to apoptosis in a p53-dependent or -independent manner (26, 27); with or without induction of p21 $^{waf1/cip1}$ (26) or p27$^{kip1}$ (28); and through caspase-1 (27), caspase-7 (29), caspase-8 (30), and/or Fas ligand (31). Loss of p53 activity is common in breast cancer (32). Nonetheless, many breast cancers are initially responsive to cytotoxic drugs and hormones (1, 33), implying that drug-induced apoptosis likely occurs through both p53-dependent and -independent mechanisms. TAM-

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induced growth arrest can occur independently of p53 (34), but the precise signaling responsible for these effects requires additional study. The primary mechanisms of cell growth arrest and apoptosis for ICI 182,780 and the importance of signaling through p53 are unknown.

In addition to our previous study implicating IRF-1 in affecting antiestrogen responsiveness in MCF-7 cells (19), Harroch *et al.* (35) observed that interleukin 6 inhibited proliferation and induced IRF-1 mRNA and IRF-1 binding to its target DNA sequence in T47D cells. In an immunohistochemistry study of IRF-1 expression in breast cancer, the authors report less IRF-1 expression in neoplastic compared with normal human breast, consistent with reduced expression of a putative tumor suppressor gene. However, IRF-1 expression was not assessed in association with established prognostic markers or clinical outcome (36).

In this study, we used the ER− MDA-MB-231 cells as a model of *de novo* antiestrogen resistance (6). To model *de novo* antiestrogen sensitivity, we used the estrogen-dependent, ER+ MCF-7 (37) and T47D cells (38) and the ER+, antiestrogen-sensitive but estrogen-independent MCF-7 variant MCF7/LCC1 (39). As a model of acquired ICI 182,780 resistance, we studied the MCF7/LCC9 cells, which were derived from MCF7/LCC1 cells and are ER+, estrogen independent, and ICI 182,780 and TAM cross-resistant (40). These studies strongly implicate signaling through IRF-1 and its protein partners as a critical mediator of antiestrogen signaling and as a key gene in a broader gene network (15, 19, 41). Hence, we now show that IRF-1 mRNA expression is induced by ICI 182,780 and repressed by estrogens in antiestrogen-sensitive cells. Hormonal regulation of IRF-1 is absent in ER− cells and is specifically lost in ER+ cells with acquired antiestrogen resistance. Both MCF-7 and T47D breast cancer cells expressing a dominant-negative IRF-1 (dnIRF-1) exhibit a decrease in sensitivity to ICI 182,780. The data separate the proapoptotic activity of ICI 182,780 from its ability to induce cell cycle arrest and are consistent with IRF-1 playing a critical role in those proapoptotic activities of antiestrogens most likely to contribute to their ability to increase overall survival and to reduce the risk of developing ER+ breast cancer (1, 16, 42).

## MATERIALS AND METHODS

**Cell Culture and Reagents.** MCF-7, T47D, ZR-75-1 (ER+, estrogen dependent, antiestrogen sensitive), and MDA-MB-231 (ER−, estrogen independent, antiestrogen unresponsive) cells were routinely grown in improved minimal essential medium (IMEM; Biofluids, Rockville, MD) with phenol red and supplemented with 5% fetal bovine serum. MCF-7 cells were originally obtained from Dr. Marvin Rich (Michigan Cancer Foundation, Detroit, MI). T47D, ZR-75-1, and MDA-MB-231 cells were obtained from the Lombardi Comprehensive Cancer Center's Tissue Culture Shared Resource. MCF-7/LCC1 (ER+, estrogen independent, antiestrogen sensitive, MCF-7 variant; Refs. 39 and 43) and MCF-7/LCC9 cells (ER+, estrogen independent, TAM and ICI 182,780 cross-resistant, MCF-7 variant derived directly from MCF7/LCC1 by selection against ICI 182,780; Refs. 19 and 40) were routinely grown in IMEM without phenol red and supplemented with 5% charcoal stripped calf serum-IMEM. All cells were maintained in a humidified incubator at 37°C in an atmosphere containing 95% air:5% $CO_2$. The steroidal antiestrogen ICI 182,780 (Faslodex; Fulvestrant) was kindly provided by Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, Cheshire, United Kingdom). Recombinant human IFN-γ was purchased from Boehringer Mannheim (Mannheim, Germany).

**RNA Extraction.** Total RNA was extracted from proliferating subconfluent cells using the TRIazol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. In brief, cells were rinsed with 1× PBS to remove serum and lysed by the addition of the TRIazol reagent. RNA was isolated by chloroform extraction and precipitated using isopropanol. Total RNA was quantified based on the absorbance at 260 nM using a spectrophotometer (DU640; Beckman, Fullerton, CA).

**Riboprobe Generation and RNase Protection Analysis.** The IRF-1 riboprobe was generated by reverse transcriptase-PCR amplification of a portion of the IRF-1 mRNA from MCF-7 cells. Amplification by PCR applied one 95°C cycle for 3 min, 40 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 1 min followed by 1 cycle of 72°C for 5 min, using the following primers: forward, TCCACCTCTCACCAAGAACC (bp 533–552), and reverse, TTCCCTTCCTCATCCTCATC (bp 873–892). To control for equivalent RNA loading, we measured expression of the constitutively expressed 36B4 mRNA that encodes the human acidic ribosomal protein P0 (44). The 36B4 riboprobe was generated as described previously (39).

RNase protection assays were performed as described previously (19, 45). In brief, plasmids were linearized by digestion with *EcoR1* and transcribed with either SP6 (IRF-1) or T7 polymerase (36B4) in the presence of [$^{32}$P]UTP. To obtain signals of approximately comparable intensity, the 36B4 riboprobes were labeled with approximately one-fifth of the [$^{32}$P]UTP concentration used to label the IRF-1 riboprobes. The IRF-1 and 36B4 riboprobes respectively generate 360- and 220-bp protected fragments. For each assay, 30 μg of total RNA was hybridized to $5 \times 10^4$ dpm of probe for 12–16 h at 50°C and digested with 40 μg/ml RNase A for 30 min at 25°C. Digestion was terminated by the addition of proteinase K (0.25 mg/ml) and 0.5% (w/v) SDS. Samples were extracted into phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated in ethanol, and the pellets were boiled in loading buffer and fractionated in 6% Tris-borate EDTA-urea polyacrylamide gels. Radioactivity was detected by autoradiography and quantified using PhosphorImager analysis (445SI; Molecular Dynamics, Sunnyvale, CA).

**Cell Lysis and Immunoblotting.** For the determination of IRF-1 protein expression, cells were seeded into 6-well dishes at $2 \times 10^5$ cells/well and cultured in normal growth media for 24 h. To examine the induction of IRF-1 in response to ICI 182,780 or estradiol, cells were seeded at $10^5$ cells/well 1 day before treatment with either 1 nM 17β-estradiol or 100 nM ICI 182,780 for 3 days. Cells were lysed in modified radioimmune precipitation assay buffer [150 mM NaCl, 50 mM Tris, 1% Igepal CA-630, and 0.5% deoxycholate (pH 7.5)] supplemented with Complete Mini protease inhibitor mixture tablets (Roche, Mannheim, Germany). Lysates were clarified by centrifugation, and equal volumes were added to 2× Laemmli sample buffer before boiling and loading onto precast 12% acrylamide gels (NuPAGE Electrophoresis System, Invitrogen, Carlsbad, CA). Proteins were transferred to nitrocellulose membranes and incubated with primary antibody (IRF-1 C-20 at 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) in Tris Buffered Saline and Tween-20 [TBST; 10 mM Tris HCl, 150 mM NaCl, and 0.05% Tween-20 (pH 8.0)] containing 5% nonfat dry milk overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature followed by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and exposure to film (X-OMAT Blue XB-1; Kodak, Rochester, NY). To confirm equal loading, membranes were reprobed as described above using a β-actin monoclonal antibody (1:5000; Sigma, St. Louis, MO).

**Generation of dnIRF-1.** Although small interfering RNA (siRNA) can be a powerful method to inhibit RNAs, we did not use this method to block IRF-1 because of the recent reports of a marked induction of an IFN response when these molecules are introduced into cells (46, 47). Thus, the best remaining approach was the use of a stably expressed dominant-negative strategy. A wild-type IRF-1 cDNA (kindly provided by Dr. Taniguchi, University of Tokyo, Japan) was subcloned into the *Xho*I site of the pGEM7Z expression vector (Promega, Madison, WI) and linearized with *Bgl*II. The dnIRF-1 comprises the full-length wild-type IRF-1 cDNA with a deletion of bp 647-1173 and contains both the 3′ and 5′ untranslated regions, the DNA-binding domain, and the nuclear localization sequences of IRF-1. We constructed dnIRF-1 by PCR amplification using bp 630–647 (TAGCAGGGCCCCTGG) as the forward primers and bp 1173–1187 (ATCAGAGAAGGTATCAGG) as the reverse primers. PCR conditions were 30 cycles of 95°C for 30 s, 45°C for 45 s, and 72°C for 5 min. Integrity of the dnIRF-1 sequence was confirmed by standard dideoxy-mediated chain-termination sequencing. dnIRF-1 was subcloned into both the *Xho*I site of the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA) and into the *Xho*I site of the pbiEGFP-tet vector [a plasmid expressing enhanced green fluorescent protein (EGFP)] under the control of a bidirectional tetracycline-responsive promoter (Clontech, Palo

Alto, CA). The IRF-1 riboprobe (above) identifies dnIRF-1, generating a 115-bp protected dnIRF-1 fragment.

**Transient Transfections and Luciferase Reporter Assay.** Cells were transfected using the FuGENE 6 method (Roche Diagnostics, Indianapolis, IN). For reporter assays, $8 \times 10^4$ cells/well were plated in 12-well plates and allowed to grow for 24 h before transfection. Cells were cotransfected with the pISRE-luc plasmid (contains five copies of the IFN-stimulated response element; ISRE) as provided in the PathDetect kit (Promega) and with either a standard control comprising a pcDNA3 plasmid without the ISRE or a pcDNA3 plasmid containing the cDNA from either IRF-1 or dnIRF-1. To control for transfection efficiency, a pRL-SV40 plasmid (Promega) containing the *Renilla* luciferase gene under the control of a constitutive SV40 promoter also was cotransfected into cells. One $\mu$g of plasmid DNA was added to serum-free media containing the FuGENE 6 reagent and allowed to incubate for 30 min at room temperature. Where appropriate, cells were maintained in growth media with or without 500 IU/ml IFN-$\gamma$ for 24–48 h. Subsequently, cells were lysed, and activation of the ISRE-luciferase construct was measured using the Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions. Luminescence was quantified using a Lumat LB 9501 luminometer (EG&G Berthold, Bundoora, Victoria, Australia).

**Stable Transfection with dnIRF-1.** For stable transfections, cells were plated in T-75 cm$^2$ plastic tissue culture flasks at a density of $0.5 \times 10^6$ cells/flask and grown for 24 h before transfection. A total of 8 $\mu$g of plasmid DNA were transfected into MCF-7 cells stably transfected with the tetR protein (MCF-7/VP16; generously provided by Dr. Susan Conrad, Michigan State University, East Lansing, MI) or T47D cells using the FuGENE6 method (above). Cells were transfected with either an empty pBI-EGFP-tet plasmid containing the EGFP selectable marker (Clontech) or one containing the dnIRF-1 cDNA and the pBABE plasmid (kindly provided by Dr. Matthew Ellis, Washington University, St. Louis, MO) encoding for puromycin resistance. Stably transfected cells were selected for growth in the presence of 1 $\mu$g/ml puromycin. Puromycin-resistant colonies expressing EGFP as measured by standard fluorescence activated cell sorting (FACS) were expanded and screened for expression of the dnIRF-1 by RNase protection. All of the transfectants used in these experiments were from pooled populations. Cells transfected with the empty control vector were designated MCF7/ctrl and T47D/ctrl and those transfected with the dnIRF-1 were designated MCF7/dnIRF-1 and T47D/dnIRF-1.

**Cell Proliferation.** MCF-7/ctrl, T47D/ctrl, MCF7/dnIRF-1, and T47D/dnIRF-1 cells were sorted aseptically by FACS in the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource for EGFP expression and plated into 12-well plastic tissue culture plates at a density of $1.5 \times 10^3$ MCF-7 or $2 \times 10^3$ T47D cells/well. Twenty-four h post plating, cells were treated with 100 nM or 1 $\mu$M ICI 182,780 or vehicle control for 72 h. Cells were then trypsinized, resuspended in PBS, and counted in a Beckman Coulter counter (Beckman Coulter Corp., Fullerton, CA) to assess cellular proliferation.

**Cell Cycle Analyses.** Cells stably transfected with the dnIRF-1 or empty control plasmids were plated in T-75 cm$^2$ plastic tissue culture flasks at a concentration of $0.5 \times 10^6$ and allowed to grow for 3 days. Cells were then analyzed for alterations in cell cycle via FACS. FACS analysis was conducted by the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource, according to the method of Vindelov *et al.* (48).

**Apoptosis.** Staining for annexin V, an optimal assay for detecting apoptosis in MCF-7 cells (49, 50), was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Control- or dnIRF-1-transfected MCF-7 cells ($1 \times 10^6$) were seeded in T-75 cm$^2$ plastic tissue culture dishes and allowed to grow for 24 h. Cells were then treated with ICI 182,780 or vehicle for 72 h, trypsinized, and pelleted by centrifugation. Cell pellets were rinsed twice in ice-cold PBS and stained with 5 $\mu$g/ml 7-aminoactinomycin D for 15 min at room temperature. After staining with 7-aminoactinomycin D, cells were washed and resuspended in annexin V binding buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, 140 mM NaCl, and 2.5 mM $CaCl_2$ (pH 7.4)]. Cells ($1 \times 10^5$) were then stained with 0.3 $\mu$g of phycoerythrin-conjugated annexin V in the dark, and flow cytometric analysis was performed using a FACStar$^{Plus}$ flow cytometer (Becton-Dickinson, Mountain View, CA) to determine the proportion of apoptotic cells in each sample. Apoptosis was measured only in cells expressing the dnIRF-1 transgene or empty vector (as assessed by concurrent EGFP expression).

**Statistical Methods.** Student's *t* test was used to compare two groups in which the data are normally distributed; a Wilcoxon *t* test was used to compare groups in which data are not normally distributed. For multiple comparisons, ANOVA was used with a *post hoc t* test for multiple comparisons. Where several experimental groups were compared with the same control, we used Dunnett's test (51).

## RESULTS

**IRF-1 Is Differentially Expressed in Antiestrogen-Sensitive and -Resistant Breast Cancer Cells.** We measured basal expression of IRF-1 mRNA by RNase protection analyses in two models of antiestrogen resistance: MCF-7/LCC9 cells (acquired resistance, ER+, ICI 182,780 and TAM cross-resistant) and MDA-MB-231 (*de novo* resistance, ER−, ICI 182,780 and TAM cross-resistant; Ref. 6). MCF-7/LCC9 cells exhibit a 4.2-fold ($P < 0.05$) and 2.6-fold ($P \leq 0.01$) lower expression of IRF-1 mRNA than the antiestrogen-sensitive MCF-7 and MCF-7/LCC1 cells, respectively (Fig. 1, *A* and *B*). IRF-1 mRNA expression in MCF-7/LCC9 cells is not significantly different from that in ER− MDA-MB-231 cells.

The half-life of the IRF-1 protein is less than 30 min, and changes in mRNA levels appear closely associated both with changes in IRF-1 protein expression and IRF-1 transcriptional activity as measured using an ISRE-based promoter-reporter assay (52). To confirm this association, we performed immunoblotting for the cells lines shown in Fig. 1, *A* and *B*. The data in Fig. 1*C* show that, as expected, the mRNA and protein levels are comparable. The MCF-7/LCC1 and MCF-7/LCC9 cells compared here are derived from the same parental MCF-7 cell line and have similar transfection efficiencies as assessed by transfection with $\beta$-galactosidase (not shown). Activity of the cotransfected *Renilla* construct (constitutively active) was used to correct for any minor differences in transfection efficiency. MCF-7/LCC9 cells exhibit 18-fold lower basal ISRE activity than their immediate parental cells MCF-7/LCC1 ($P \leq 0.01$; Fig. 1*D*). These data reflect the differential IRF-1 mRNA expression that we have previously detected in gene expression microarrays (19) and now confirm by RNase protection analyses (Fig. 1, *A* and *B*) and immunoblot (Fig. 1*C*). These data also confirm that IRF-1 mRNA expression, protein expression, and transcriptional activation are closely related in breast cancer cells. Furthermore, these data show that IRF-1 expression and activity are significantly lower in ER+ and ER− antiestrogen-resistant cells compared with antiestrogen-sensitive cells.

**IRF-1 mRNA Expression Is Regulated through ER in Breast Cancer Cells.** Data on basal expression in the sensitive and resistant cells imply that estrogens and antiestrogens may affect IRF-1 mRNA expression. Fig. 2, *A* and *B*, shows the ability of 100 nM ICI 182,780, a clinically relevant concentration (53), to induce IRF-1 mRNA in the three best characterized and most widely used ER+ human breast cancer cell lines (6): MCF-7 ($P \leq 0.001$); T47D ($P \leq 0.001$); and ZR-75-1 ($P < 0.05$). This effect appears to be ER mediated, because IRF-1 mRNA induction by ICI 182,780 is blocked in the presence of estradiol in MCF-7 cells (Fig. 2, *C* and *D*) and ICI 182,780 is unable to induce IRF-1 in the ER− MDA-MB-231 cells (Fig. 2, *A* and *B*).

The dose-response relationships for the endocrine regulation of IRF-1 mRNA in MCF-7 and T47D cells were determined. Cells were plated and 24 h later treated with various doses of ICI 182,780 or 0.1% (v/v) ethanol vehicle for 72–96 h before RNA isolation. ICI 182,780 induces IRF-1 mRNA in a dose-dependent manner, with a maximal 2.5-fold induction at a dose of 100 nM in both MCF-7 (Fig. 3, *A* and *B*; $P \leq 0.001$) and T47D (Fig. 3, *C* and *D*; $P \leq 0.01$) cells. In contrast, IRF-1 mRNA expression is significantly down-regulated in response to treatment with 1 nM estradiol (Fig. 3, *E* and *F*; $P \leq 0.001$).

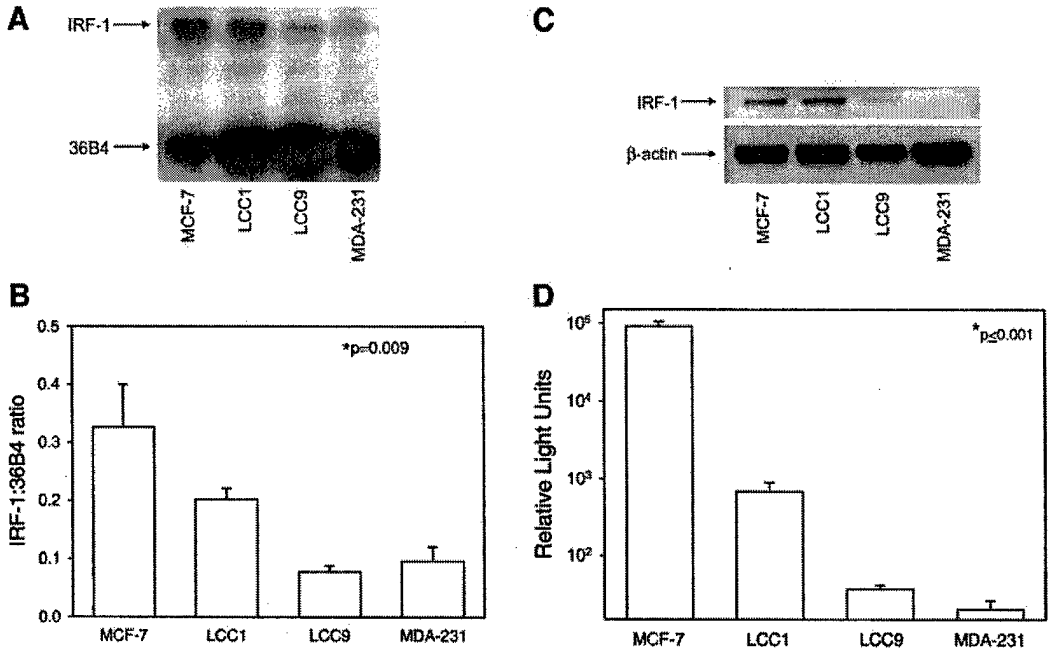


Fig. 1. Basal IRF-1 mRNA and protein expression and transcriptional activation in breast cancer cell lines. *A*, representative RNase protection assay. *36B4*, loading control. *B*, IRF-1 mRNA expression measured by RNase protection and presented as mean ± SE of three determinations, in which intensity is expressed as a ratio of IRF-1:36B4 (ANOVA, $P = 0.009$; *, $P < 0.05$ MCF-7 *versus* MCF7/LCC9; $P < 0.01$ MCF-7/LCC1 *versus* MCF7/LCC9; $P = 0.528$ MDA-MB-231 *versus* MCF7/LCC9). *C*, representative immunoblot of IRF-1 protein. *β-actin*, loading control. *D*, basal transcriptional activity of IRF-1 in breast cancer cell lines (ISRE-luc promoter-reporter assay). Data represent mean ± SE of four determinations and are presented as relative light units. ANOVA, $P < 0.01$; *, $P \leq 0.001$ for MCF-7 *versus* each of MCF7/LCC1, MCF7/LCC9, and MDA-MB-231. *, $P$ = NS for MCF7/LCC9 *versus* MDA-MB-231.

**ER-Mediated Regulation of IRF-1 Is Specifically Lost in Anti-estrogen-Resistant Cells.** Although the data in Fig. 2 and Fig. 3 show the regulation of IRF-1 mRNA expression, this would likely be of limited functional relevance if similar patterns of regulation occur in antiestrogen-resistant cells. However, the ability of both estrogen and ICI 182,780 to regulate IRF-1 mRNA expression is lost in the MCF-7/LCC9 cells (Fig. 4, *A–D*), and ICI 182,780 is unable to regulate IRF-1 expression in the antiestrogen-resistant ER− cell line, MDA-MB-231 (Fig. 2, *A* and *B*). In contrast, the estrogen-independent but antiestrogen-sensitive MCF7/LCC1 cells retain the ability of estrogen to inhibit IRF-1 mRNA expression (Fig. 4, *A* and *B*). Consistent with the data in Fig. 1, we found similar changes in the hormonal regulation of IRF-1 protein in immunoblots (not shown). MCF7/LCC1 cells do not require estrogens to grow *in vitro* or *in vivo* (estrogen independent) but retain some estrogen responsiveness (40, 43). Thus, the apparent loss of ER-mediated regulation of IRF-1 is associated with acquired antiestrogen resistance but not estrogen independence.

We then asked whether the specificity of endocrine regulation of IRF-1 that is lost in antiestrogen-resistant cells also extended to nonhormonal inducers of IRF-1. In mouse embryonic fibroblasts, IRF-1 is induced by treatment with the cytotoxic drug doxorubicin (26), one of the most active single cytotoxic drugs used in the treatment of breast cancer (54, 55). MCF-7 cells induce IRF-1 mRNA in response to treatment with doxorubicin (Fig. 5, *A* and *B*). Importantly, MCF-7/LCC9 cells retain their ability to regulate IRF-1 expression in response to 1 μM doxorubicin (increase by over 7-fold; $P \leq 0.001$), a response also shared by MDA-MB-231 cells (increase by over 4-fold; $P < 0.05$; Fig. 6, *A* and *B*). Thus, antiestrogen resistance is associated with a specific, ER-mediated change in the regulation of IRF-1 expression, rather than a global loss of IRF-1 mRNA regulation. This specificity allows cells to retain the ability to induce IRF-1 and undergo an IRF-1-regulated apoptotic cell death in response to other cytotoxic agents.

**ICI 182,780-Induced Inhibition of Cell Proliferation Is Reduced by dnIRF-1.** To study the functional relevance of changes in IRF-1 activity in affecting antiestrogen responsiveness, we created dnIRF-1. dnIRF-1 contains the IRF-1 DNA binding domain and nuclear localization signal but lacks the protein binding and transcriptional activation domains. Dominant-negative activity of dnIRF-1 was apparent in its ability to inhibit basal ISRE activity, activity induced by transient expression of wild-type IRF-1, and IFN-γ stimulated ISRE activity in MCF-7 (Fig. 7; all comparisons $P < 0.001$; Student's *t* test). Control cells transfected with an empty expression vector exhibit no regulation of ISRE activity; control cells transfected with empty luc vector (no ISRE) showed no activity (not shown). Importantly, we did not want dnIRF-1 to eliminate all IRF-1 transcriptional activity but rather to inhibit activity to an extent broadly equivalent to that induced by antiestrogens. Complete loss of IRF-1 could induce compensatory responses of uncertain biological relevance, and there is no evidence that IRF-1 expression is fully lost in breast tumors (36). Because IRF-1 mRNA expression levels are closely related to ISRE activity in breast cells (Fig. 1), the data in Fig. 7 imply that dnIRF-1 inhibits a level of ISRE activity broadly comparable with that induced by antiestrogens in sensitive cells (Fig. 2; Fig. 3).

dnIRF-1 was stably transfected into both MCF-7 and T47D cells, and the ability of dnIRF-1 to affect ICI 182,780-induced inhibition of cell proliferation was measured (Fig. 8, *A* and *B*). In cell proliferation assays, expression of dnIRF-1 significantly reduced responsiveness of the MCF-7/dnIRF-1 and T47D/dnIRF-1 transfectants to ICI 182,780. At 100 nM, the dose that maximally induces IRF-1 mRNA expression, MCF-7/dnIRF-1 and T47D/dnIRF-1 transfectants were significantly less sensitive to ICI 182,780 compared with their respective controls. Similar differences in the responsiveness of MCF-7/dnIRF-1 and T47D/dnIRF-1 cells were seen at 1 μM ICI 182,780 ($P = 0.002$, MCF-7 100 nM ICI 182,780; $P = 0.013$, MCF-7 1 μM ICI 182,780; $P = 0.002$, T47D 100 nM ICI 182,780; $P = 0.043$, T47D 1 μM ICI 182,780; Student's *t* test; Fig. 8, *A* and *B*).

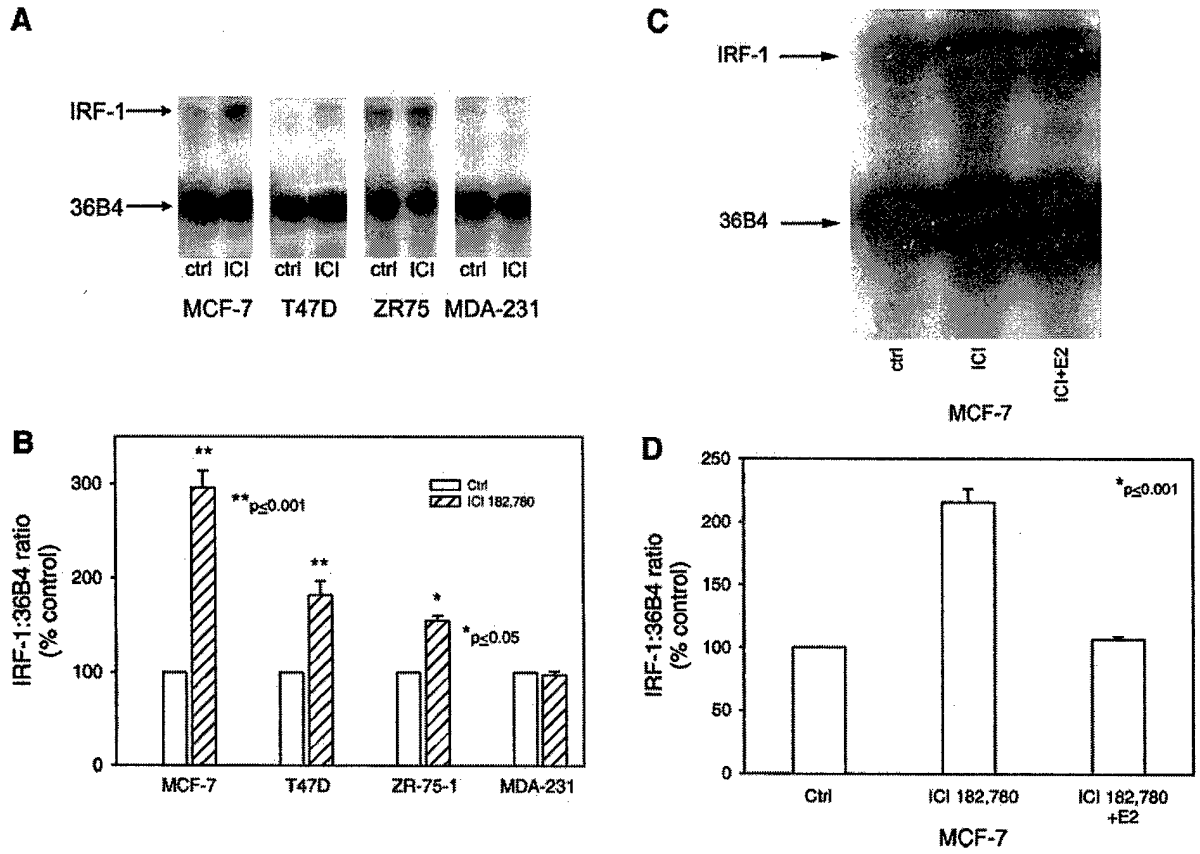


Fig. 2. Endocrine regulation of IRF-1 mRNA transcription in breast cancer cell lines. *A*, representative RNase protection data for IRF-1 mRNA expression. *B*, ICI 182,780 induces IRF-1 mRNA expression in ER+ but not ER− breast cancer cells. Cells were treated with 100 nM ICI 182,780 or ethanol vehicle. Data represent mean ± SE of three determinations, in which absorbance is expressed as a ratio of IRF-1:36B4 and represented as a percentage of vehicle control-treated cells. *, $P \leq 0.001$ for ICI 182,780 *versus* control-treated cells within each cell line for MCF-7 and T47D and $P < 0.05$ for ZR75. $P = 0.880$ for MDA-MB-231; Student's *t* test. *C*, representative RNase protection data for IRF-1 mRNA expression; *D*, estradiol reverses ICI 182,780-induced IRF-1 mRNA transcription in MCF-7 cells. *, $P < 0.001$; Student's *t* test.

**dnIRF-1 Does Not Affect ICI 182,780-Induced Changes in Cell Cycle Distribution.** Although antiestrogens can affect both cell cycle distribution and the rate of apoptosis, cell proliferation assays measure the sum of these activities. Hence, we asked directly whether the effects of dnIRF-1 on proliferation reflected an inhibition of the ability of ICI 182,780 to arrest cells in $G_0$-$G_1$. The data in Fig. 9 show that dnIRF-1 does not affect the ICI 182,780-induced cell cycle arrest in $G_0$-$G_1$ in either MCF-7/dnIRF-1 (Fig. 9*A*) or T47D/dnIRF-1 cells (Fig. 9*B*). Thus, the residual antiproliferative effects of ICI 182,780 (Fig. 8) in dnIRF-1-expressing cells are those conferred by cell cycle arrest. These data strongly implicate changes in apoptosis as being the primary mechanism through which dnIRF-1 reduces the antiproliferative effects of ICI 182,780 in MCF-7 and T47D cells.

**ICI 182,780-Induced Apoptosis Is Reduced by dnIRF-1.** To determine whether the effects of dnIRF-1 on cellular sensitivity to ICI 182,780 are mediated by its ability to influence signaling to apoptosis, the ability of dnIRF-1 to affect ICI 182,780-induced apoptosis was assessed directly by measuring annexin V staining (49, 50). Apoptosis was measured only in those cells expressing the dnIRF-1 transgene or empty vector control (as assessed by EGFP expression), to ensure that any effects were likely to be a direct result of the inhibition of IRF-1. When treated with 100 nM ICI 182,780, 30% of MCF-7 control transfectants undergo apoptosis. Expression of dnIRF-1 significantly reduces this ICI 182,780-induced apoptotic response by >4-fold in MCF-7/dnIRF-1 cells to 7% (Fig. 10; $P < 0.034$). The basal rate of apoptosis, measured in control MCF-7 cells treated with ethanol, is about 5% (Fig. 10). Thus, the full apoptotic response to ICI 182,780 is blocked by dnIRF-1. Studies were also performed in T47D and T47D/dnIRF-1 cells, which unlike MCF-7 cells contain a mutant and nonfunctional p53. T47D/dnIRF-1 cells show a similar 4-fold reduction in the ability of ICI 182,780 to induce apoptosis (not shown). These data with dnIRF-1 in both MCF-7 (wild-type p53) and T47D (mutant p53) strongly suggesting that IRF-1 is a critical mediator of this signal and that its activities are independent of p53.

## DISCUSSION

Data from our previous studies demonstrate an association between IRF-1 expression and acquired cross-resistance to antiestrogens (19). We now show that IRF-1 is a key signaling protein involved in mediating the sensitivity of breast cancer cells to ICI 182,780-induced apoptosis. Basal IRF-1 mRNA expression is down-regulated in antiestrogen-resistant cells (both the ER+ MCF7/LCC9 model of acquired resistance and the ER− MDA-MB-231 model of *de novo* resistance). Moreover, the ability of antiestrogens to regulate IRF-1 mRNA transcription is absent in the ER− and lost in the ER+ antiestrogen-resistant cells. The functional relevance of these observations is shown by the ability of dnIRF-1 to reduce significantly the antiproliferative effects of ICI 182,780 in both the antiestrogen-sensitive MCF-7 and T47D cells. Notably, dnIRF-1 does not eliminate basal IRF-1 activity in these cells. Loss of IRF-1 activity could induce confounding compensatory effects unlikely to occur in breast tumors, which appear to retain detectable IRF-1 protein expression (36). Thus, the data reported herein likely reflect the contribution of only the antiestrogen induced IRF-1.

Specificity of these effects, in the context of the signaling of IRF-1

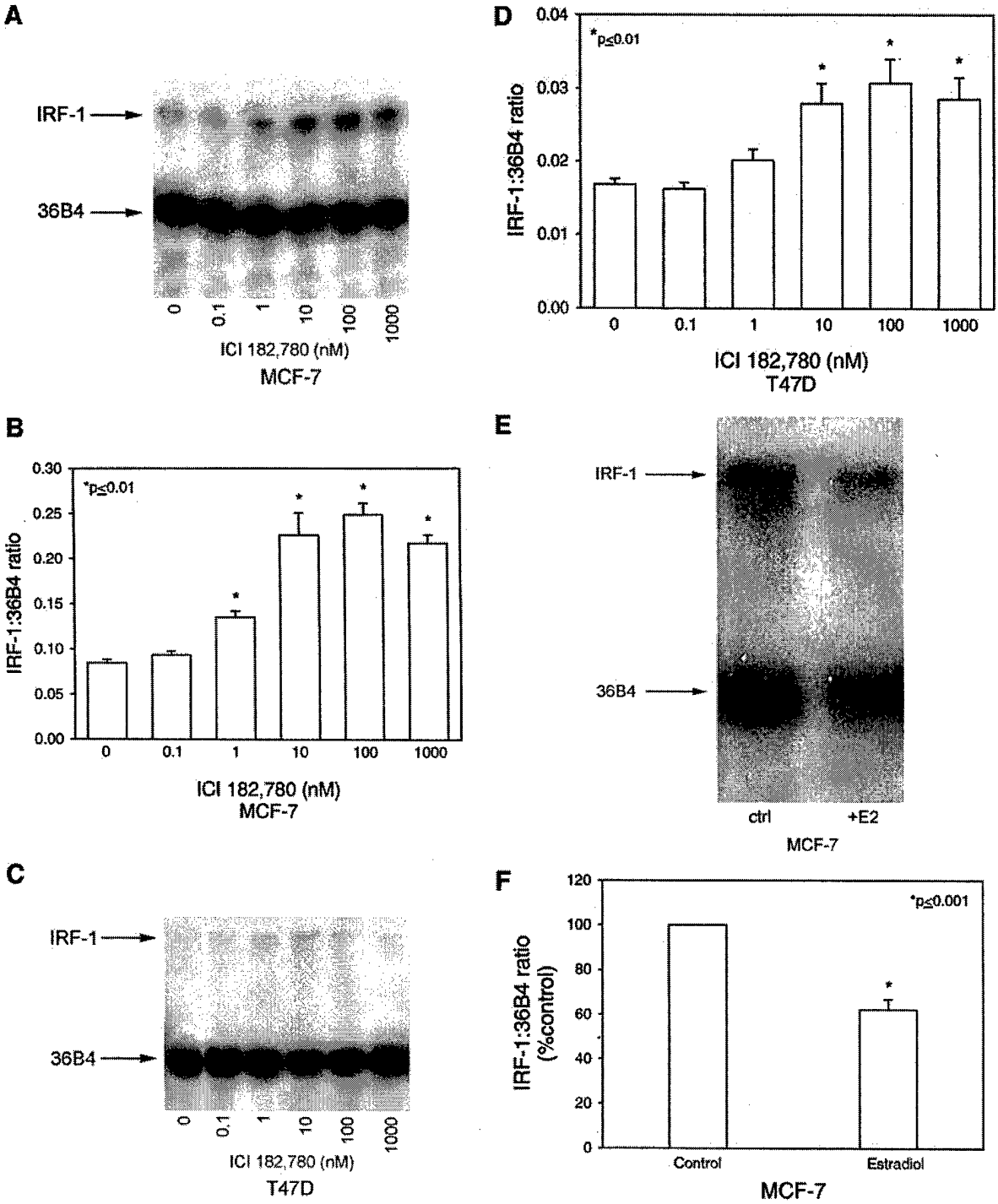


Fig. 3. Dose-response and time-response relationships for the induction of IRF-1 mRNA by ICI 182,780 in MCF-7 (*A* and *B*) and T47D cells (*C* and *D*). *A*, representative RNase protection assay. *36B4*, loading control. *B*, dose-response relationship for IRF-1 mRNA induction in MCF-7 cells. Cells were treated with ICI 182,780 or ethanol vehicle for 72 h. Data represent mean ± SE of three independent replicate experiments, in which absorbance is expressed as a ratio of IRF-1:36B4. *, $P \leq 0.01$ for treatments *versus* control; Dunnett's test. *C*, representative RNase protection assay. *36B4*, loading control. *D*, dose-response relationship for IRF-1 mRNA induction in T47D cells. Cells were treated as in *B*. Data represent mean ± SE of three independent replicate experiments, in which absorbance is expressed as a ratio of IRF-1:36B4. *, $P \leq 0.01$ for treatments *versus* control; Dunnett's test. *E*, representative RNase protection assay. *36B4*, loading control. *F*, estradiol regulation of IRF-1 mRNA expression in MCF-7 cells. Cells were stripped of estrogens, grown in the absence of estrogen (charcoal stripped calf serum-IMEM), and then treated with either 1 nM estradiol or ethanol vehicle for 24 h. Data represent mean ± SE of three independent replicate experiments, in which absorbance is expressed as a ratio of IRF-1:36B4 and represented as a percentage of vehicle control-treated cells. *, $P < 0.001$; Student's *t* test.

in response to ER-mediated events, also is apparent. Estradiol blocks antiestrogen-induced IRF-1 in MCF-7 cells, and no endocrine regulation is seen in ER− cells. Neither the loss of endocrine regulation in MCF7/LCC9 cells nor the absence of its endocrine regulation in MDA-MB-231 cells compromises the ability of doxorubicin to induce IRF-1 in these cells and to inhibit their proliferation (not shown). This latter effect is consistent with patterns of clinical responses to these drugs, because breast cancer patients who are resistant to antiestrogens can respond to cytotoxic chemotherapy.

The ability of dnIRF-1 to block ICI 182,780-induced inhibition of

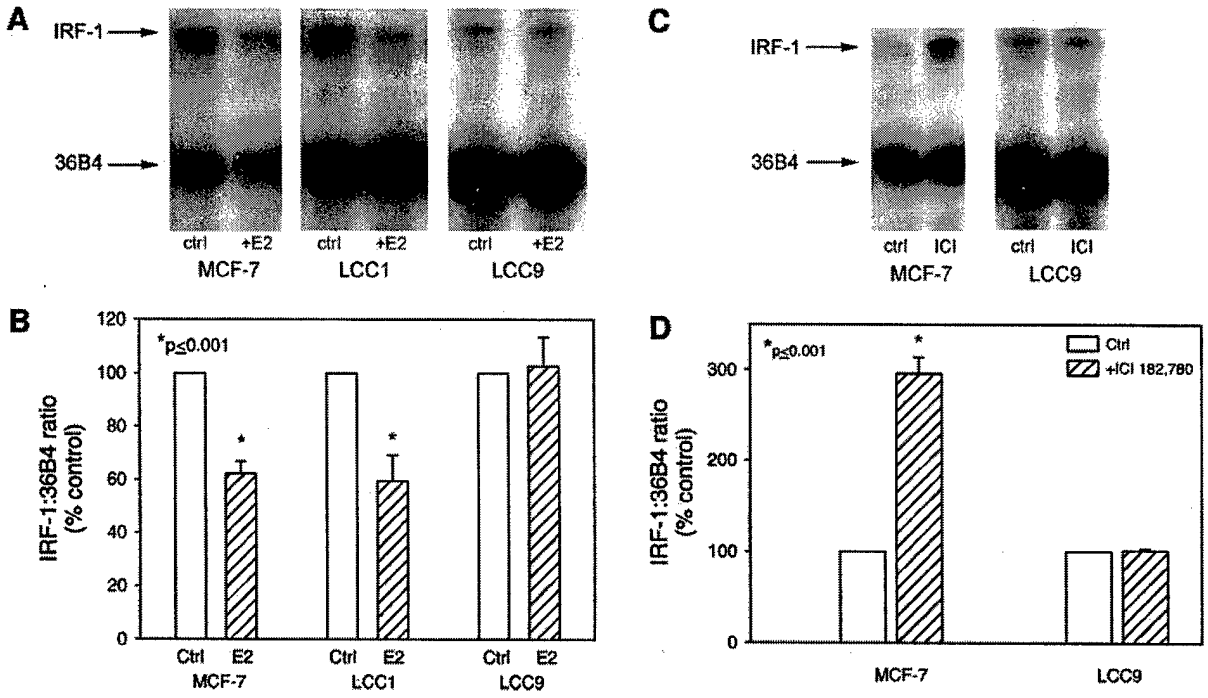


Fig. 4. Hormonal regulation of IRF-1 expression is lost in antiestrogen-resistant cells. *A*, representative RNase protection assay. *36B4*, loading control. *B*, estradiol regulation of IRF-1 mRNA expression. Cells were stripped of estrogens, grown in the absence of estrogen (charcoal stripped calf serum-IMEM), and then treated with either 1 nM estradiol or ethanol vehicle for 24 h. Data represent mean ± SE of three independent replicate experiments, in which absorbance is expressed as a ratio of IRF-1:36B4 and represented as a percentage of vehicle control-treated cells. *, $P \leq 0.001$ for estradiol *versus* control for each of MCF7 and MCF7/LCC1 cells; $P = 0.864$ for estradiol *versus* control-treated MCF-7/LCC9 cells; Student's *t* test. *C*, ICI 182,780 regulation of IRF-1 mRNA expression; representative RNase protection assay. *36B4*, loading control. Cells were treated with 100 nM ICI 182,780 or ethanol vehicle for 72 h. *D*, ICI 182,780 regulation of IRF-1 mRNA expression. Data represent mean ± SE of three determinations, in which absorbance is expressed as a ratio of IRF-1:36B4 and represented as a percentage of vehicle control-treated cells. *, $P \leq 0.001$ for ICI 182,780 *versus* control-treated MCF-7 cells; $P = 0.971$ for ICI 182,780 *versus* control-treated MCF7/LCC9 cells; Student's *t* test.

cell proliferation in MCF-7/dnIRF-1 and T47D/dnIRF-1 cells could reflect changes in the effects of ICI 182,780 on cell cycle and/or apoptosis. However, dnIRF-1 does not affect ICI 182,780-induced cell cycle arrest when expressed in either MCF-7 or T47D cells. In marked contrast, dnIRF-1 effectively eliminates ICI 182,780-induced apoptosis in both MCF-7/dnIRF-1 and T47D/dnIRF-1 cells. Thus, we can separate cell cycle arrest from apoptosis and attribute a significant component of antiestrogen-induced apoptotic signaling to IRF-1. Because dnIRF-1 abrogates ICI 182,780-induced apoptosis (Fig. 10) while enhancing cell growth by approximately 50% (Fig. 8), apoptosis and cell cycle arrest likely contribute equally to the apparent antiproliferative effects of ICI 182,780.

Functionally separating antiestrogen-induced apoptosis from antiestrogen-induced growth arrest has several important implications. Novel therapeutic approaches designed to increase the proapoptotic effects of antiestrogens may be an effective means to improve their ability to increase overall survival in patients because this should increase the proportion of cells undergoing apoptotic cell death. Modalities that increase only the ability of antiestrogens to induce a cell cycle arrest will likely be a less effective strategy. For example, many arrested cells will survive and thereby have more opportunities to adapt, acquire resistance, and generate subsequent disease recurrence. Measuring basal IRF-1 expression and/or the ability of an antiestrogen to induce IRF-1 in the neoadjuvant setting may improve the prediction of endocrine responsiveness. Currently, we incorrectly predict antiestrogen sensitivity in 66% of ER+/progesterone receptor-negative, 55% of ER−/progesterone receptor-positive, and 25% of ER+/progesterone receptor-positive tumors (6).

The ability of IRF-1 to induce growth arrest is associated with the induction of various genes including p53-dependent and -independent events (26, 27) and interactions that may include $p21^{waf1/cip1}$ (26).

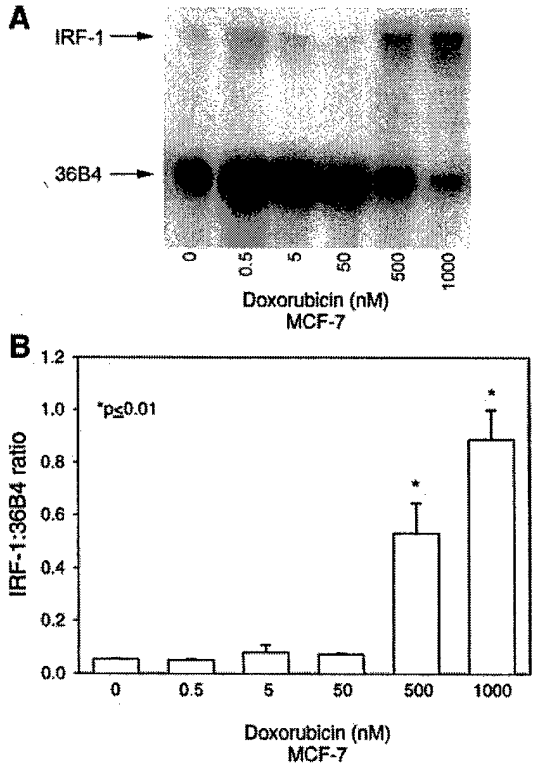


Fig. 5. Doxorubicin induction of IRF-1 mRNA expression. *A*, representative RNase protection assay. *36B4*, loading control. *B*, MCF-7 cells were treated with 1 μM doxorubicin. Data represent mean ± SE of three determinations, in which absorbance is expressed as a ratio of IRF-1:36B4. *, $P \leq 0.01$; Dunnett's test.

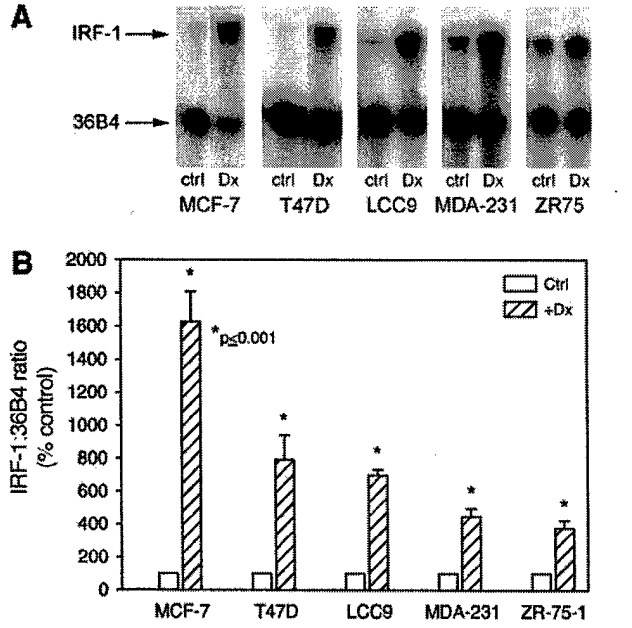


Fig. 6. Doxorubicin induces IRF-1 mRNA expression in both ER+ and ER− breast cancer cell lines. RNase protection assay of ER+ MCF-7, T47D, MCF-7/LCC9, and ZR75 cells and ER− MDA-MB-231 cells treated with 1 μM doxorubicin (*DX*) for 24 h. *A*, representative data from RNase protection assay; *B*, graphical representation of RNase protection assays of ER+ and ER− breast cancer cell lines treated with doxorubicin. Data represent mean ± SE of three independent determinations, in which absorbance is expressed as a ratio of IRF-1:36B4 and represented as a percentage of vehicle control-treated cells. **, $P \leq 0.001$ for doxorubicin *versus* control-treated cells within each cell line for MCF-7 and MCF-7/LCC9 cells; *, $P \leq 0.05$ for MDA-MB-231 cells; Student's *t* test.

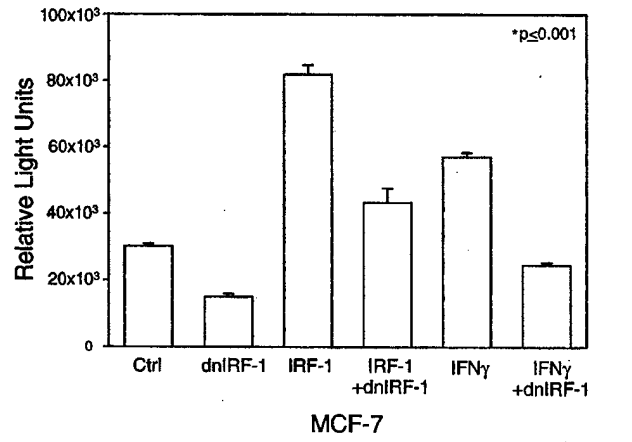


Fig. 7. Inhibition of basal and IFN-γ stimulated ISRE activity by dnIRF-1 in MCF-7 cells. Data represent mean ± SE (representative experiment of four independent replicates), in which data are represented by relative light units. Where appropriate, MCF-7 cells were treated with 500 IU of IFN-γ. *, $P \leq 0.001$ for all transfections *versus* control; Dunnett's test.

Interactions requiring both $p21^{waf1/cip1}$ and p53 may not be central components in antiestrogen signaling through IRF-1. Nonetheless, preliminary data suggest an increase in $p21^{waf1/cip1}$ mRNA expression after ICI 182,780 treatment in MCF-7 and T47D cells (2.91 ± 0.89-fold), consistent with both a previous report on $p21^{waf1/cip1}$ regulation by ICI 182,780 (56) and activation of IRF-1. MCF-7 cells express wild-type p53, whereas T47D cells express a mutant and nonfunctional p53 (57), but both cell lines are responsive to antiestrogen-induced apoptosis in a manner that remains sensitive to the effects of dnIRF-1. However, a role for $p53/p21^{waf1/cip1}$ signaling in the cell cycle effects of antiestrogens cannot be excluded.

The ability of ICI 182,780 to induce apoptosis through IRF-1 activity is likely mediated through changes in caspase activation. IRF-1 can induce several caspases (27, 29, 30), and inhibition of caspase activity blocks antiestrogen-induced apoptosis (58). A specific requirement for caspase-3 seems unlikely because this caspase is not expressed in MCF-7 cells (59). IRF-1 signaling through caspase-1 (27), caspase-7 (29), and caspase-8 (30) is strongly implicated. For example, IRF-1 induces caspase-1 (60), which can regulate apoptosis in normal mammary epithelial cells (61). Overexpression of caspase-1 is lethal in MCF-7 cells (62). Caspase-7 is expressed in MCF-7 cells and may substitute for the loss of caspase-3 in these cells (63). IFN-γ, which induces IRF-1 activity in MCF-7 cells (Fig. 7), is reported to sensitize both MCF-7 and MDA-MB-231 cells to apoptosis through inducing caspase-8 (64). TAM can induce caspase-8 (58), and consistent with our observations in T47D cells, caspase-8-induced apoptosis occurs independent of p53 (64). Studies to determine which caspases are functionally involved in IRF-1 signaling in breast cancer are currently in progress.

Although data in this study show reduced IRF-1 expression and loss of its endocrine regulation in antiestrogen-resistant cells, the level of IRF-1 activity in cells is also affected by protein-protein interactions with the nucleolar phosphoprotein nucleophosmin (NPM; Ref. 65). Not previously reported in breast cancer cells, we now have preliminary data to suggest that this functional interaction also occurs in T47D cells (not shown). NPM is an estrogen-induced protein in

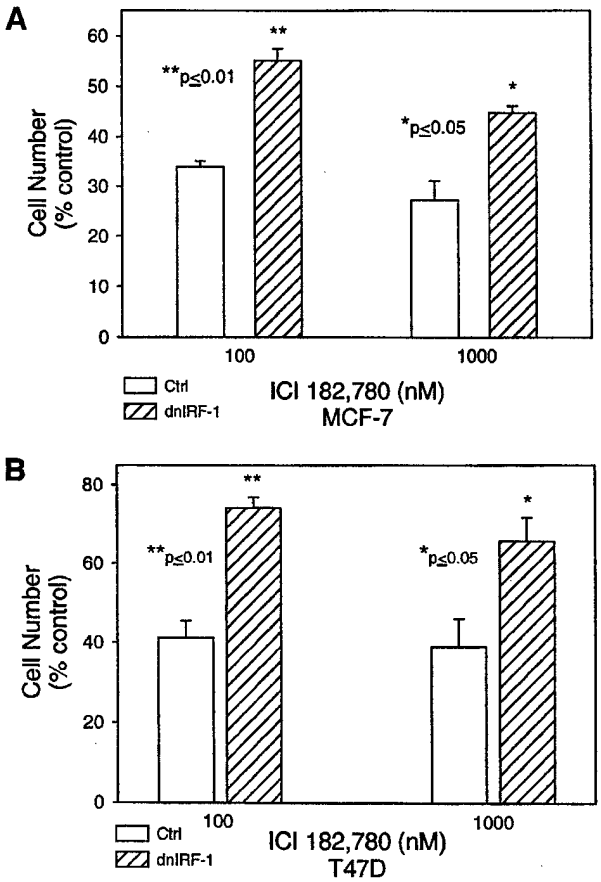


Fig. 8. Inhibition of ICI 182,780 effects on cell proliferation by dnIRF-1. *A*, MCF-7 cells with and without constitutive dnIRF-1 expression. *B*, T47D cells with and without constitutive dnIRF-1 expression. For each cell line, cells were treated with ethanol vehicle, 100 nM ICI 182,780 or 1 μM ICI 182,780 for 3 days. Data represent mean ± SE of three determinations. *, $P = 0.002$, MCF-7 100 nM ICI 182,780; $P = 0.013$, MCF-7 1 μM ICI 182,780; Student's *t* test. $P = 0.002$, T47D 100 nM ICI 182,780; $P = 0.043$, T47D 1 μM ICI 182,780; Student's *t* test.

MCF-7 cells that is down-regulated by antiestrogens (66), and its expression is increased in MCF-7/LCC9 when compared with MCF-7/LCC1 cells (19). Thus, in addition to down-regulating IRF-1 mRNA expression, antiestrogen-resistant cells have up-regulated expression of an endogenous inhibitor (NPM). Interestingly, we have previously shown that NPM autoantibody levels are lower in TAM-treated patients, suggesting that NPM/IRF-1 interactions also may be clinically relevant (67).

It seems likely that an acquired antiestrogen resistance phenotype is conferred not by the alteration of a single gene or signal transduction pathway but rather through the perturbation of a signaling network of integrated signaling pathways (15). The data presented here are consistent with cell signaling through IRF-1 being a key component or node in such a signaling network. Activity as a signaling node is implied by (*a*) the potential for diversity/redundancy of signaling to a key end point (apoptosis) downstream of IRF-1 (cooperation with p53, $p21^{waf1/cip1}$, and regulation of several caspases); (*b*) the redundancy apparent in regulating IRF-1 activity (down-regulation of basal transcription, loss of ER-mediated transcription, and concurrent up-regulation of the endogenous inhibitor NPM); (*c*) the apparent specificity for antiestrogen resistance (cytotoxic drugs can induce IRF-1 in antiestrogen-resistant cells); and (*d*) the altered regulation of IRF-1 activity/expression in models of both *de novo* and acquired antiestrogen resistance. This node may be important in affecting other signals in breast cancer cells. For example, IRF-1 is downstream of tumor necrosis factor signaling, and both tumor necrosis factor $\alpha$ and its receptor tumor necrosis factor R1 are down-regulated in MCF7/LCC9 cells, implying a cross-resistance to tumor necrosis factor-mediated events (15, 19).

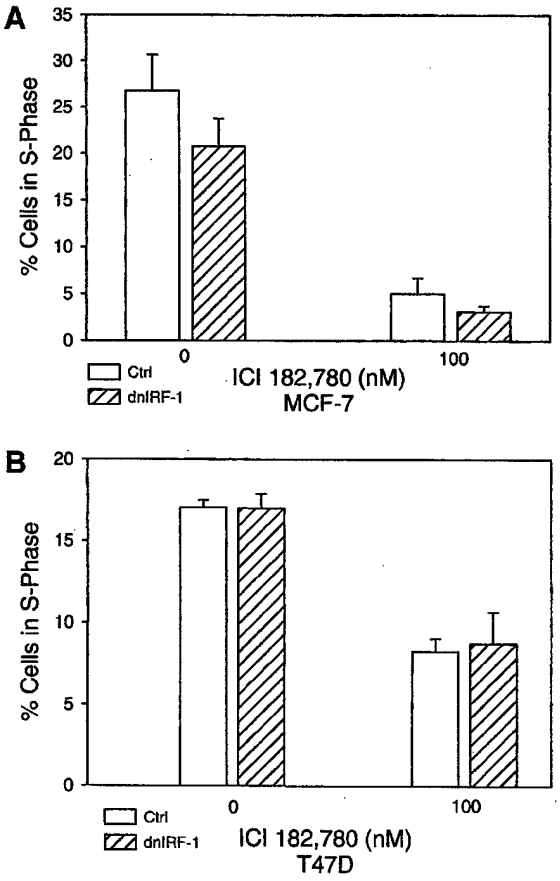


Fig. 9. Expression of dnIRF-1 does not affect ICI 182,780-induced changes in cell cycle distribution. *A*, MCF-7 cells with and without constitutive dnIRF-1 expression; *B*, T47D cells with and without constitutive dnIRF-1 expression. For each cell line, cells were treated with either ethanol vehicle or 100 nM ICI 182,780 for 3 days. Data represent mean ± SE of three determinations. *, $P = 0.40$, MCF-7 0 nM ICI 182,780 (vehicle control); $P = 0.237$, MCF-7 100 nM ICI 182,780; Student's *t* test. $P = 0.962$, T47D 0 nM ICI 182,780; $P = 0.836$, T47D 100 nM; Student's *t* test.

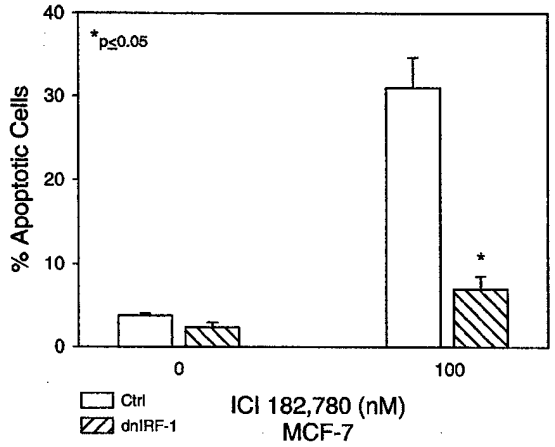


Fig. 10. ICI 182,780-induced apoptosis is reduced by dnIRF-1 expression. MCF-7 cells with and without constitutive dnIRF-1 expression. Data represent mean ± SE of three independent replicate experiments. dnIRF-1- and control-transfected cells were treated with either ethanol vehicle or 100 nM ICI 182,780 for 3 days. Annexin V analysis was measured by FACS. *, $P \leq 0.05$ for ICI 182,780-treated control transfectants *versus* dnIRF-1 transfectants (Dunnett's test).

The ability of doxorubicin to induce IRF-1 in antiestrogen-resistant cells and to inhibit proliferation in these cells is a clinically relevant phenotype. We and others (36, 68) have detected IRF-1 expression by immunohistochemistry in breast cancer specimens, this pattern of expression being consistent with a potential tumor suppressor role for IRF-1 (21, 36). Our preliminary data suggest that the pattern of IRF-1 expression in breast cancers, as measured by immunohistochemistry, is consistent with other components of our network. For example, we detect an inverse pattern of expression between IRF-1 and nuclear factor $\kappa$B as seen in MCF7/LCC1 *versus* MCF7/LCC9 cells (19, 68). These observations may ultimately lead to a better ability to identify patients that will respond to antiestrogens and to predict which patients will ultimately develop antiestrogen resistance. Interfering with the putative "IRF-1 node" may allow for the development of novel therapeutic strategies in endocrine-resistant breast cancers.

## ACKNOWLEDGMENTS

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# The NFκB inhibitor parthenolide restores ICI 182,780 (Faslodex; Fulvestrant)-induced apoptosis in antiestrogen-resistant breast cancer cells

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***Abbreviations:*** CCS-IMEM, improved minimal essential medium supplemented with 5% calf charcoal stripped serum; ER, estrogen receptor-alpha; ICI, ICI 182,780 or Faslodex; IκB, inhibitor of NFκB; IKK, IκB kinase.

***Keywords:*** NFκB, Faslodex, parthenolide, cell cycle, proliferation, apoptosis, breast cancer.

***Running title:*** Parthenolide restores antiestrogen-induced apoptosis

## Abstract

The molecular mechanisms underlying the acquisition of resistance to the antiestrogen Faslodex are poorly understood, although enhanced expression and activity of nuclear factor kappa B (NFκB) have been implicated as a critical element of this phenotype. The purpose of this study was to elucidate the mechanism by which NFκB upregulation contributes to Faslodex resistance and to determine whether pharmacological inhibition of NFκB by the small molecule parthenolide could restore Faslodex-mediated suppression of cell growth. Basal expression of multiple NFκB-related molecules in MCF7-derived LCC1 (antiestrogen-sensitive) and LCC9 (antiestrogen-resistant) breast cancer cells was determined, and cells were treated with Faslodex or parthenolide. The effect of these drugs either singly or in combination was assessed by cell proliferation, estrogen receptor (ER)-dependent transcriptional activation, cell cycle analysis, and apoptosis assays. Expression of the p65 NFκB subunit and the upstream NFκB regulator IKKγ/NEMO were increased in the resistant MCF7/LCC9 cells ($p=0.001$ and 0.04, respectively). While MCF7/LCC9 cells were unresponsive to Faslodex alone, parthenolide effectively inhibited MCF7/LCC9 cell proliferation and the combination of Faslodex and parthenolide resulted in a 4-fold synergistic reduction in cell growth ($p=0.03$). This corresponded to a restoration of Faslodex-induced apoptosis ($p=0.001$), with no observable changes in ER-dependent transcription or cell cycle phase distribution. Since parthenolide has shown safety in Phase I clinical trials, these findings have direct clinical relevance and provide support for the design of clinical studies combining antiestrogens and NFκB inhibitors such as parthenolide in ER-positive breast cancer.

# Introduction

Antiestrogens inhibit the function of the estrogen receptor (ER), a nuclear transcription factor that directs the expression of genes that contribute to proliferation and cell growth (reviewed in 1,2). The most frequently prescribed is the nonsteroidal antiestrogen Tamoxifen, which has been shown to be highly effective in both the treatment of ER-positive breast tumors and in reducing breast cancer incidence in women at high risk for the disease. However, most ER-positive tumors become estrogen-independent and develop resistance to antiestrogen therapy, while the remainder (~30%) exhibit *de novo* or intrinsic resistance. Once resistance has developed, treatment with most nonsteroidal antiestrogens is usually unsuccessful.

In contrast, the steroidal antiestrogen Faslodex (ICI 182,780; ICI) induces significant clinical responses in patients whose tumors have acquired Tamoxifen resistance (3). The effectiveness of Faslodex in patients with Tamoxifen-resistant disease is similar to that of the aromatase inhibitor anastrozole, and several clinical trials have shown that Faslodex may be a viable alternative to nonsteroidal antiestrogens and aromatase inhibitors as a first-line endocrine treatment (4). Faslodex stimulates degradation of the ER and prevents receptor dimerization, inhibiting estrogen-dependent gene transcription (5,6). As a pure antagonist of the ER, Faslodex is not associated with the increased risk for endometrial cancer that is seen with Tamoxifen (7).

The antiestrogen resistance phenotype is complex, involving many changes at the cellular and molecular levels. Antiestrogens are cytostatic, inducing a G0/G1 block in breast cancer cells in culture (1,8). However, these drugs are also capable of actively inducing programmed cell death or apoptosis, which is consistent with the ability of antiestrogens to increase overall survival (9). One way in which breast cancer cells may become antiestrogen resistant is through

changes in gene networks that control cell proliferation and apoptosis (10). To test this hypothesis, we developed several variant cell lines from the estrogen-dependent and antiestrogen sensitive MCF-7 breast cancer cells (11,12). MCF7/LCC1 cells are estrogen-independent but remain responsive to antiestrogens; MCF7/LCC9 cells are derivatives of MCF7/LCC1 that have acquired resistance to Faslodex.

Several genes were found to be altered in the resistant MCF7/LCC9 cells, when their transcriptomes were compared with that of their antiestrogen sensitive MCF7/LCC1 parental cells by serial analysis of gene expression (SAGE) and microarray analysis (10). For example, we implicated loss of the putative tumor suppressor interferon regulatory factor-1 (IRF-1) in acquired resistance and have recently shown IRF-1 to be a key mediator of the proapoptotic effects of Faslodex in MCF-7 cells (13).

Altered expression of the p65/RelA member of the NFκB transcription factor family, which can form functional heterodimers with IRF-1 (14), also was strongly implicated in acquired Faslodex resistance. mRNA levels of p65/RelA are upregulated two-fold in the MCF7/LCC9 cells, NFκB-dependent transcription are increased ten-fold, and MCF7/LCC9 cells exhibit a greater sensitivity to the growth inhibitory effects of parthenolide, a small molecule inhibitor of NFκB (10). These data strongly but indirectly implicate NFκB action in acquired antiestrogen resistance.

The NFκB family contains five members that form dimers and regulate the transcription of various genes including cytokines, cell adhesion molecules, the pro-proliferative proteins c-myc and cyclin D1, and several inhibitors of apoptosis (15). Inhibitors of the NFκB pathway show promise as anticancer and anti-inflammatory agents (16). Parthenolide, a sesquiterpene

lactone that was first isolated from the feverfew herb (*Tanacetum parthenium*) native to Central America (17), is a relatively specific small molecule inhibitor of NFκB (18). Parthenolide and other members of the sesquiterpene lactone class have garnered recent attention as promising candidates for cancer treatment either as single agents or in combination with other cytotoxic drugs (19,20). For example, parthenolide has anti-inflammatory, anticancer, and antiangiogenic properties, and has successfully undergone Phase I/II clinical trials (21,22).

Constitutive NFκB activity is widely observed in many tumor types (23), including breast cancer where it is associated with resistance to apoptosis-inducing agents (24). In many tumor lines, autocrine secretion of cytokines and growth factors has recently been implicated in the constitutive activation of NFκB (25). Importantly, NFκB activity also increases in breast cancer cells as they acquire the ability to grow in the absence of estrogen (26,27). These findings strongly implicate NFκB signaling in the control of breast cancer cell growth and response to antiestrogens.

In this study, we sought to clarify the mechanism by which NFκB upregulation may affect resistance to Faslodex and determine whether pharmacological inhibition of NFκB could restore sensitivity to the drug. We show here that in addition to p65/RelA, expression of the upstream regulator NEMO/IKKγ is also increased in the resistant cells. The NFκB inhibitor parthenolide efficiently inhibits cell growth and restores sensitivity to Faslodex by synergistically enhancing apoptosis. Our data indicate that inhibition of NFkB may be a successful approach in the treatment of ER-positive breast cancers that have acquired resistance to antiestrogen therapy. NFκB inhibition also may reduce the incidence or delay the onset of

antiestrogen resistance. These data provide support for considering the design of clinical studies combining antiestrogens and NFκB inhibitors such as parthenolide in ER+ breast cancer.

## Materials and Methods

*Cell Culture and Reagents*: MCF-7-derived MCF7/LCC1 and MCF7/LCC9 cells (11,12) were routinely cultured in phenol red-free improved minimal essential media (IMEM; Biofluids, Rockville, MD) supplemented with 5% charcoal-stripped calf serum (CCS) (CCS-IMEM). Cells were maintained in a humidified atmosphere at 37°C and 95% air/5% $CO_2$. 17β-estradiol (estradiol, E2) and parthenolide were purchased from Sigma (St. Louis, MO), and ICI 182,780 (ICI, Faslodex) was a kind gift of Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK).

*Cell Lysis, Immunoblotting, and Immunoprecipitation*: Cells were grown in either 10 $cm^2$ dishes or T-75 $cm^2$ tissue culture flasks prior to lysis. To determine the effects of parthenolide and ICI 182,780 on protein expression, cells were treated with vehicle, 100 nM ICI 182,780 (the $IC_{50}$ for the control/parental LCC1 cells), or 600 nM parthenolide singly or in combination in CCS-IMEM for 72 hours. Cells were then lysed in modified radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% Igepal CA-630, and 0.5% deoxycholate) supplemented with Complete Mini protease inhibitor cocktail tablets (Roche, Mannheim, Germany) and 1 mM sodium orthovanadate phosphatase inhibitor (Sigma). Lysates were clarified by centrifugation and total protein was quantitated using the bicinchoninic acid (BCA) assay purchased from Pierce (Rockford, IL). 20 μg of whole cell lysate were resolved by polyacrylamide gel electrophoresis using NuPAGE 12% pre-cast gels (Invitrogen, Carlsbad, CA). Proteins were then transferred to nitrocellulose membranes, which were probed with the following antibodies overnight at 4°C: p65 NFκB sc-109 (1:800; Santa Cruz Biotechnology,

Santa Cruz, CA), p50 NFκB sc-8414 (1:200; Santa Cruz), p52 NFκB (1:200; Upstate Biotechnology, Charlottesville, VA), IKKγ/NEMO sc-8330 (1:200; Santa Cruz), IκBα sc-371 (1:200; Santa Cruz), phospho-Akt (Ser473) (1:1000, Cell Signaling, Beverly, MA), or Akt (1:1000, Cell Signaling). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ) for one hour at room temperature prior to enhanced chemiluminescence (Amersham Biosciences) and exposure to film. To confirm equal loading of the gels, membranes were reprobed with antibodies for β–actin (1:5000; Sigma).

For immunoprecipitations, 400 μg of cell lysate were incubated with 2.5 μl of p65 NFκB antibodies overnight at 4°C with rotation. The following day, 30 μl of Protein A-Sepharose beads (Amersham) were added for one hour at 4°C to recover the immune complexes, which were then washed twice in modified RIPA, twice in Tris-saline (TN; 50 mM Tris pH 7.5, 150 mM NaCl), and resuspended in 2X Laemmli sample buffer prior to electrophoresis as described above.

*Cell Proliferation Assays*: MCF7/LCC1 and MCF7/LCC9 cells were seeded at a density of 1-2x$10^4$ cells per well in 24-well plates, and 24 hours later were treated with the indicated concentrations of drug in CCS-IMEM. Cells were incubated with the drugs for 7 days, and the media were changed on days 3 and 5. Cells were then trypsinized, resuspended in phosphate-buffered saline (PBS; Biofluids), and counted using a Z1 Single Coulter Counter (Beckman/Coulter, Miami, FL). At least three independent experiments were performed in

quadruplicate, and data were normalized to vehicle-treated cells. Data are presented as the mean ± standard error (S.E.) for a representative experiment.

*Transcriptional Reporter Assays*: The estrogen response element-containing 3xERE-tk-luc reporter plasmid was purchased from Promega (Madison, WI). MCF7/LCC1 and MCF7/LCC9 cells were seeded into 12-well plates at a density of 7-8x$10^4$ cells per well. The following day, cells were transfected with 0.4 μg of luciferase reporter plasmid and 0.1 μg pCMV-Renilla (Promega) per well using the FuGENE 6 transfection reagent (Roche, Indianapolis, IN). Three hours post-transfection, media were changed and cells were treated with 100 nM ICI 182,780 and/or 600 nM parthenolide in CCS-IMEM for 24 hours. Subsequently, cells were lysed and activation of the luciferase constructs was measured using the Dual Luciferase Assay Kit (Promega). Luminescence was quantified using a Lumat LB 9501 luminometer (EG&G Berthold, Bundoora VIC, Australia). Luciferase values were normalized to Renilla luminescence, and four independent experiments were performed each at least in quadruplicate. Data are presented as the mean ± S.E. for all experiments.

*Cell Cycle Assays*: 5x$10^5$ cells were seeded into 10 $cm^2$ dishes one day prior to treatment with 100 nM ICI 182,780 and/or 600 nM parthenolide in CCS-IMEM for 24 hours. Cells were then analyzed for alterations in cell cycle via fluorescence activated cell sorting, which was performed by the Lombardi Comprehensive Cancer Center Flow Cytometry Shared Resource according to the method of Vindelov *et al.* (28). Data are presented as the mean ± S.E. for three independent experiments.

*Apoptosis Assays*: $1x10^5$ cells were seeded onto 18x18 mm glass coverslips in each well of a 6-well plate in duplicate and the following day were treated with 100 nM ICI 182,780 and/or 600 nM parthenolide in CCS-IMEM for 24 hours. Cells were then fixed with 3.7% formalin in PBS for 20 minutes at room temperature prior to annexin V and propidium iodide (PI) staining using the Vybrant Apoptosis Assay Kit #3 purchased from Vector Laboratories (Burlingame, CA). Coverslips were then mounted on glass slides using VectaShield fluorescence mounting medium (Vector Laboratories). Cells were visualized on a Nikon E600 fluorescence microscope (provided by the Lombardi Comprehensive Cancer Center Microscopy Shared Resource), and several random fields ($\geq$ 200 cells) were scored per treatment condition. The number of cells stained red (PI, indicating necrosis) was subtracted from the number of cells stained green (annexin V-FITC, indicating apoptosis), and subsequently divided by the total number of cells seen by phase-contrast. Data are presented as the percentage of apoptotic cells and represent the mean $\pm$ S.E. for three independent experiments.

*Statistical Analyses*: Two-tailed Student's t tests were used for the comparison of two groups for immunoblot, cell proliferation, and apoptosis assays as indicated. For luciferase reporter assays, Dunn's post hoc t-test was used to compare all treatment groups following one-way analysis of variance (ANOVA). Defining the nature of the interaction between Faslodex and parthenolide was performed by determining the R index (RI) (29). RI values were obtained by calculating the expected cell survival ($S_{exp}$; the product of survival obtained with drug A alone and the survival obtained with drug B alone) and dividing $S_{exp}$ by the observed cell survival in the presence of

both drugs ($S_{obs}$). A $S_{exp}/S_{obs}$ value greater than 1.0 indicates a synergistic interaction. This method is an appropriate way to define synergy in this case because clinically relevant concentrations of Faslodex are ineffective on cell proliferation or apoptosis in the resistant MCF7/LCC9 cells when given as a single agent (29).

## Results

**p65/RelA and NEMO/IKKγ are upregulated in antiestrogen-resistant cells.**

Our previous studies identified a two-fold upregulation of p65/RelA mRNA in Faslodex resistant MCF7/LCC9 cells by expression microarray analysis (10). To confirm altered expression of p65 at the protein level and to examine other NFκB family members and regulatory molecules, whole cell lysates were prepared from MCF7/LCC9 cells and antiestrogen-sensitive MCF7/LCC1 cells and subjected to SDS-PAGE and immunoblot analysis. Similar to mRNA levels, p65/RelA protein is increased approximately two-fold in the MCF7/LCC9 cells (Fig. 1A, $p=0.001$). In contrast, expression of the p50 subunit of the NFκB heterodimer (Fig. 1B, $p=0.35$) or of p52 NFκB2 (data not shown) is not different between the cell lines.

Transcriptional activity of the p65/p50 heterodimer is modulated by the inhibitor IκB, which is in turn negatively regulated by the IκB kinase (IKK) complex comprised of IKKα, IKKβ, and the scaffolding protein NFκB essential modulator (NEMO/IKKγ) (30). To determine whether MCF7/LCC9 cells exhibit changes in these regulatory molecules, lysates were immunoblotted for NEMO/IKKγ and IκBα (Figs. 1C and 1D). While there is no significant change in IκBα expression ($p=0.10$), a significant two-fold increase in the level of NEMO/IKKγ is apparent in MCF7/LCC9 cells ($p=0.04$). NEMO/IKKγ is required for activity of the IKK complex and the inhibitory phosphorylation of IκB in response to inflammatory stimuli that activate NFκB (31), and dysregulation of NEMO is linked to several human pathologies (32). These data suggest that NEMO may also play a role in the response of breast cancer to antiestrogens.

To examine whether the binding of p65 and p50 was altered in antiestrogen-resistant cells, cell lysates were immunoprecipitated with p65 antibodies and immune complexes were captured and subjected to SDS-PAGE as described above (Fig. 1E). No clear differences in p65/p50 complex formation were found between MCF7/LCC1 and MCF7/LCC9 cells.

Independent of the IKK-IκB signaling pathway, NFκB can also be activated by phosphatidylinositol 3-kinase (PI3K); PI3K-mediated activation of Akt can enhance NFκB transcriptional activity without the degradation of IκB (33). Because overexpression of active Akt has also been shown to induce resistance to antiestrogens and cytotoxic drugs (34), MCF7/LCC1 and MCF7/LCC9 cell lysates described above were immunoblotted for phospho-Serine 473 Akt (Fig. 1F). No difference in the level of activated phospho-Akt is observed in the antiestrogen-resistant MCF7/LCC9 cells, suggesting that Akt→NFκB signaling is not the only pathway through which cells can modulate NFκB activation and acquire resistance to Faslodex.

**Inhibition of NFκB by Parthenolide restores Faslodex sensitivity to MCF7/LCC9 cells.**

We have previously reported that MCF7/LCC9 cells are more sensitive than MCF7/LCC1 cells to growth inhibition by parthenolide, suggesting that these cells, in which p65/RelA is upregulated, are more dependent on NFκB-driven cell growth (10). 100 nM Faslodex approximates the $IC_{50}$ for proliferation in antiestrogen-sensitive MCF7/LCC1 cells but is ineffective in MCF7/LCC9 cells (Figure 2A, $p=0.01$). To determine whether inhibition of NFκB activity could restore Faslodex sensitivity, MCF7/LCC9 cells were treated with increasing concentrations of parthenolide in the presence or absence of 100 nM Faslodex. In the absence of

Faslodex, parthenolide effectively inhibits MCF7/LCC9 cell proliferation with an $IC_{50}$ of 500-600 nM. However, the addition of Faslodex generates a significant nearly 5-fold sensitization, where 50% growth inhibition occurs at a concentration of 100 nM parthenolide (Figure 2B; p=0.034 for parthenolide plus Faslodex compared to parthenolide alone).

The interaction of Faslodex and parthenolide is synergistic in MCF7/LCC9 cells, generating an R-index (RI) value of 1.82. Treatment with 100 nM Faslodex and 600 nM parthenolide also produces a greater than additive inhibition of cell proliferation (p=0.05, RI=1.48). These data strongly suggest that the upregulated NFκB activity present in MCF7/LCC9 cells is a major contributor to the antiestrogen resistance phenotype.

**Parthenolide and Faslodex synergistically increase apoptosis.**

We subsequently sought to define the mechanism by which parthenolide and Faslodex synergistically inhibit the growth of MCF7/LCC9 cells. A primary action of antiestrogens is to antagonize endogenous estrogen and block estrogen receptor (ER) function; Faslodex can achieve this by affecting receptor turnover (5). We asked whether parthenolide can restore Faslodex-mediated inhibition of ER-dependent transcriptional activity (Figure 3). MCF7/LCC1 and MCF7/LCC9 cells were co-transfected with an ERE-tk-luciferase reporter vector and the pCMV-Renilla control vector. Three hours post-transfection, cells were treated with estradiol, Faslodex, and parthenolide for 24 hrs before performing dual-luciferase promoter-reporter assays.

MCF7/LCC1 cells exhibit a basal ERE-luciferase activity that is enhanced 8-fold by estradiol treatment and almost abolished by Faslodex. In contrast, MCF7/LCC9 cells express a

higher basal ERE-luciferase activity that is slightly enhanced by estradiol but is not inhibited by Faslodex treatment. Parthenolide either alone or in combination with Faslodex has no statistically significant effect on ERE-luciferase activity in MCF7/LCC9 cells, suggesting that the mechanism of their antiproliferative synergy does not involve the regulation of ER-dependent transcriptional events.

Treatment with antiestrogens such as Faslodex can have a cytostatic effect on cell growth, typically manifested as an accumulation of cells in the G0/G1 phase of the cell cycle (1,8). In some cell systems, parthenolide can arrest cells at the G2/M phase transition (35). To test whether parthenolide restored the cytostatic activities of Faslodex or induced a G2/M blockade, MCF7/LCC9 cells were treated with Faslodex ± parthenolide or ethanol vehicle for 24 hrs prior to cell cycle analysis (Figure 4). Parthenolide alone or in combination with Faslodex does not alter the MCF7/LCC9 cell cycle profile, indicating that a block in cell cycle progression does not explain the synergistic reduction in cell growth.

Faslodex and other antiestrogens actively promote apoptosis, and parthenolide has been demonstrated to cooperatively enhance apoptosis induced by other cytotoxic agents such as paclitaxel and 4-hydroxyphenylretinamide (4-HPR) (19,20). Therefore, we measured the effects of Faslodex ± parthenolide or ethanol vehicle on apoptosis as detected by immunostaining for FITC-conjugated annexin V and propidium iodide (PI) staining (Table I). Approximately 3% of vehicle-treated and 4% of Faslodex-treated MCF7/LCC9 cells undergo apoptosis. In contrast, parthenolide treatment increases the apoptotic fraction to nearly 10%; upon cotreatment with Faslodex and parthenolide, 18% of the cells undergo apoptosis. Importantly, the level of apoptosis seen in the presence of the Faslodex/parthenolide combination was essentially identical

to that induced by Faslodex alone in the antiestrogen-sensitive LCC1 cells (Table I). The strong induction of apoptosis in MCF7/LCC9 cells seen in the presence of both drugs is statistically significant compared to either Faslodex or parthenolide alone ($p=0.001$ and $p=0.01$, respectively). The calculated RI value of 2.28 for the parthenolide/Faslodex interaction indicates synergistic induction of apoptosis in antiestrogen-resistant MCF7/LCC9 cells.

Parthenolide stabilizes the inhibitor IκB, leading to the retention of p65 in the cytoplasm in an inactive state (36). Therefore, we measured the effects of Faslodex ± parthenolide or ethanol vehicle on IκBα expression (Figure 5). Since protein levels of IκBα were unchanged in MCF7/LCC9 cells regardless of treatment, parthenolide may be acting through other alternative mechanisms to synergize with Faslodex and restore the apoptotic response to antiestrogen-resistant MCF7/LCC9 cells.

## Discussion

Our previous studies reported the p65/RelA subunit of NFκB as being upregulated in MCF-7-derived MCF7/LCC9 breast cancer cells that had acquired resistance to Faslodex (10,11). We have now identified additional changes in the expression of NFκB pathway members in these cells and demonstrated that pharmacological inhibition of NFκB restores Faslodex sensitivity by markedly enhancing apoptosis. Since the NFκB inhibitor parthenolide is currently being investigated in clinical trials (21), these findings have direct clinical relevance and provide support for the design of clinical studies combining antiestrogens and NFκB inhibitors such as parthenolide in ER+ breast cancer.

Protein expression of the p65/RelA subunit of NFκB is increased approximately two-fold in MCF7/LCC9 cells when compared with antiestrogen-sensitive MCF7/LCC1 cells; this agrees with the upregulation in mRNA levels previously observed (10). However, NFκB-dependent transcriptional activity is elevated almost 10-fold in MCF7/LCC9, implying that other elements of the NFκB signaling pathways are activated in these cells. We found no changes in p50 expression or association with p65; there were also no alterations in expression of p52 NFκB2 (data not shown) or the NFκB negative regulator IκBα. PI3K-dependent signaling can also activate NFκB and Akt activation, a primary downstream target of PI3K, has been implicated in antiestrogen resistance. However, we found no differences the levels of phospho-Akt, indicating that this pathway also is unlikely to account for the increased NFκB activity.

In contrast, MCF7/LCC9 cells express approximately two-fold higher levels of NEMO/IKKγ. NEMO binds to IKKβ and controls the formation of the IKK complex (37); this

is required for the activation of NFκB in response to external stimuli such as TNFα (31,38). Upregulation of NEMO in MCF7/LCC9 cells would enhance the kinase activity of IKK, and likely adds to the elevated levels of p65 to further increase basal NFκB activation.

Constitutive NFκB activity is known to arise as breast cancer cells progress to an estrogen-independent (26,27) and antiestrogen-resistant state (10). However, this is the first report implicating NEMO/IKKγ in these events. Regulatory control of NEMO is complex, involving sequential small ubiquitin-like modifier (SUMO) and ubiquitin modification occurring in both the cytoplasm and nucleus (39). Whether the hormonal regulation of NEMO is altered in the MCF7/LCC9 cells has not been determined, but is currently being pursued to clarify further the mechanism by which NFκB activity is elevated in breast cancer cells with acquired antiestrogen resistance.

The NFκB inhibitor parthenolide strongly represses the proliferation of MCF7/LCC9 cells (100 nM=ineffective; 600 nM=$IC_{50}$) and restores their sensitivity to Faslodex. For example, while treatment with 100 nM Faslodex alone is ineffective, 50% growth inhibition is achieved in the presence of only 100 nM parthenolide. This interaction between Faslodex and parthenolide, which generates an estimated R index value of 1.82, is synergistic (29). The restoration of Faslodex sensitivity by parthenolide is a significant finding and directly supports our hypothesis that the upregulated NFκB activity present in MCF7/LCC9 cells is a major contributor to the antiestrogen resistance phenotype.

We have explored several mechanisms through which parthenolide may restore Faslodex sensitivity in antiestrogen-resistant cells. For example, NFκB inhibition could rescue the ability

of Faslodex to block ER-dependent transcriptional activity. Expression of several well characterized estrogen-regulated genes, including progesterone receptor, pS2, and cathepsin D (10), is increased in MCF7/LCC9 cells. This may reflect the 7-fold higher basal levels of ER-dependent transcription in vehicle-treated MCF7/LCC9 cells, relative to that seen in MCF7/LCC1 cells. Faslodex completely inhibits transcription of an ERE-luciferase construct in MCF7/LCC1 cells (antiestrogen sensitive) but has no effect on ERE-mediated transcription in MCF7/LCC9 cells (antiestrogen resistant). While exogenous expression of p65/RelA NFκB can repress ER transcriptional activity *in vitro* (40), treatment with parthenolide does not restore the ability of Faslodex to affect ER function in MCF7/LCC9 cells. Thus, the elevated basal ER-dependent transcriptional activity in these cells is likely not due to NFκB upregulation.

Faslodex induces G0/G1 arrest in MCF7/LCC1 cells treated with 100 nM Faslodex (data not shown), but neither Faslodex nor parthenolide has any effect on MCF7/LCC9 cell cycle distribution. Parthenolide-mediated G2 arrest has been observed at 10-fold higher concentrations than were used in this study (35); therefore, the possibility that MCF7/LCC9 cell cycle progression is inhibited by much higher concentrations of parthenolide cannot be excluded. Indeed, several studies with parthenolide and other sesquiterpene lactones used 1-10 μM or greater concentrations to achieve 50% inhibition of cell growth, whereas our cells required up to 10-fold lower concentrations to achieve the same results.

Our results show that the inhibition of cell growth by Faslodex and parthenolide is not primarily cytostatic in nature. In marked contrast, a combination of Faslodex and parthenolide synergistically promotes programmed cell death (RI=2.28). Importantly, the proportion of apoptotic cells observed in the presence of both drugs (19%) is comparable to that seen when

MCF7/LCC1 cells are treated with the same dose of Faslodex (19.9%). Parthenolide can enhance the apoptotic activities of taxanes and retinoids (19,20), and we now demonstrate that it can also potentiate the death of antiestrogen-resistant breast cancer cells by restoring their sensitivity to Faslodex.

DeGraffenried et al (41) have recently reported that NFκB inhibition by parthenolide increased breast cancer cell sensitivity to Tamoxifen. However, these investigators used MCF-7 cells genetically engineered to overexpress activated Akt; these cells exhibit Tamoxifen resistance and NFκB activation that is entirely dependent on Akt-mediated pathways. In our cell system, which was derived by selection in the presence of Faslodex rather than by genetic engineering of the cells, NFκB upregulation does not correlate with enhanced Akt activity.

Sesquiterpene lactones in general, and parthenolide in particular, can prevent the degradation of IκB, block activation of IKK, alkylate cysteine-38 in p65/RelA to prevent DNA binding, and inhibit inducible nitric oxide synthase (iNOS) (36,42,43). We found no evidence of IκBα stabilization when MCF7/LCC9 cells were treated with parthenolide either in the absence or presence of Faslodex. Parthenolide can also inhibit p42/44 mitogen activated protein kinase (MAPK) activity (42) but we observed no reduction in the levels of phospho-MAPK in our cells upon parthenolide treatment (data not shown).

Our studies clearly demonstrate that treatment with the NFκB inhibitor parthenolide is a viable approach to restoring Faslodex-induced apoptosis in breast cancer cells that have acquired resistance. Several preclinical studies have shown that parthenolide also is effective in the treatment or chemoprevention of cancer cell growth (44). A Phase I study of feverfew in cancer patients was recently completed and reported no significant toxicity observed at the doses tested

(21). Other direct or indirect inhibitors of the NFκB pathway also show promise as antiproliferative agents (16) and include some nonsteroidal anti-inflammatory drugs, antioxidants, immunosuppressants, proteasome inhibitors, and glucocorticoids. Our work now demonstrates that inhibition of NFκB may also be useful for the treatment of ER-positive breast cancers that have acquired resistance to antiestrogen therapy, thus restoring the activity of one of the most active and least toxic modalities available in the treatment of endocrine-dependent breast cancer.

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**Table 1** – Faslodex and parthenolide synergistically enhance apoptosis in MCF7/LCC9 cells

| Cell Line/Drug | % apoptosis ± S.E. | p value |
|---|---|---|
| LCC1 veh | 4.22 ± 0.98 | - |
| LCC1 ICI | 19.96 ± 4.43 | 0.03* |
| LCC9 veh | 3.20 ± 1.96 | - |
| LCC9 ICI | 4.41 ± 0.90 | 0.61* |
| LCC9 parth | 9.95 ± 1.21 | 0.04* |
| LCC9 ICI+parth | 18.34 ± 1.45 | 0.003*;0.001^;0.01# |

* vs. vehicle; ^ vs. ICI; # vs. parth
RI=2.28 for combination of ICI and parthenolide

**Figure Legends**

**Figure 1.** Expression of NFκB family members and upstream regulatory molecules. **(A– D)** Quantitiation and representative immunoblots of p65/RelA, p50NFκB, NEMO/IKKγ, and IκBα levels in MCF7/LCC1 and MCF7/LCC9 cells. 20 μg of lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted as shown. β-actin=loading control. Data shown are representative of at least 3 independent experiments and are presented as the mean ± standard error (SE). p values were calculated by Student's t test. **(E)** Coimmunoprecipitation of p65 and p50. 400 μg of lysates were immunoprecipitated with polyclonal anti-p65 antibodies; immune complexes were isolated and separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted as shown. **(F)** Akt activity is not altered in antiestrogen-resistant MCF7/LCC9 cells. 20 μg of lysates were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with antibodies specific for phospho-Ser473 of Akt. The membrane was then stripped and reprobed for total Akt.

**Figure 2.** Parthenolide inhibits the proliferation of antiestrogen resistant cells and partially restores Faslodex sensitivity. **(A)** MCF7/LCC9 cells are unresponsive to Faslodex. Cells were seeded in quadruplicate and treated with 100 nM Faslodex in CCS-IMEM for 7 days prior to counting. Data are from a single representative experiment and are presented as the mean ± SE of relative proliferation (relative to vehicle-treated control) and the p value was calculated by Student's t test. The experiment was independently performed at least three times. **(B)** Faslodex and parthenolide synergistically inhibit MCF7/LCC9 cell proliferation. Cells were seeded in quadruplicate and treated with 0-6000 nM parthenolide in the presence or absence of

100 nM Faslodex in CCS-IMEM for 6 days. Data represent the mean ± SE of relative proliferation. *, p=0.034 vs. 100 nM parthenolide without Faslodex by Student's t test, RI=1.82. #, p=0.05 vs. 600 nM parthenolide without Faslodex and RI=1.48.

**Figure 3**. Combined treatment with Faslodex and parthenolide does not inhibit ER-dependent transcriptional activity. MCF7/LCC1 and MCF7/LCC9 cells were transfected in quadruplicate with ERE-tk-luciferase and pCMV-Renilla constructs prior to treatment with 10 nM estradiol, 100 nM Faslodex, and 600 nM parthenolide singly or in combination (or ethanol vehicle) in CCS-IMEM for 24 hours. Luciferase assay data are expressed as the mean ± SE of the ratio of luciferase-to-Renilla activity (relative light units) for four independent experiments. $p<0.001$ for all treatment groups by one-way ANOVA.

**Figure 4**. Combined treatment with Faslodex and parthenolide has no effect on the cell cycle profile of MCF7/LCC9 cells. Cells were treated with 100 nM Faslodex, 600 nM parthenolide, Faslodex plus parthenolide, or ethanol vehicle in CCS-IMEM for 24 hours prior to cell cycle analysis. Data represent the mean ± SE for three independent experiments and are expressed as % total cells.

**Figure 5**. Combined treatment with Faslodex and parthenolide has no effect on the stability of IκBα expression. LCC9 cells were treated with 100 nM Faslodex, 600 nM parthenolide, Faslodex plus parthenolide, or ethanol vehicle in CCS-IMEM for 24 hours prior to cell lysis. 20

μg of lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted as shown. β-actin=loading control.

Figure 1A

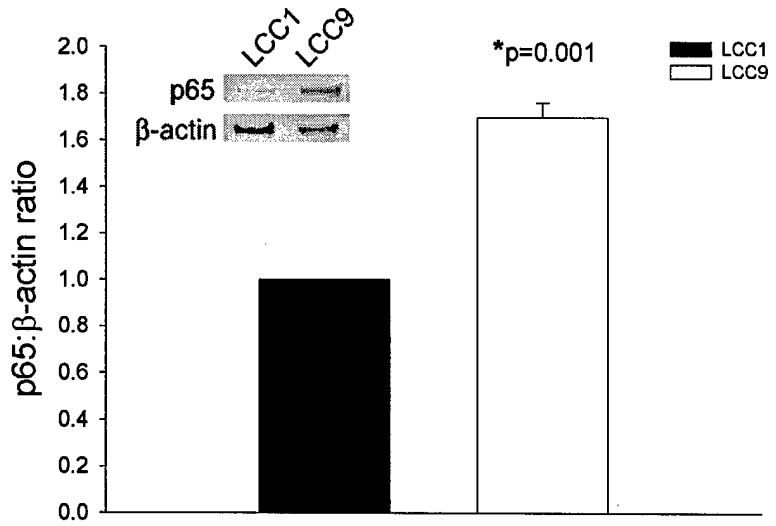



Figure 1B

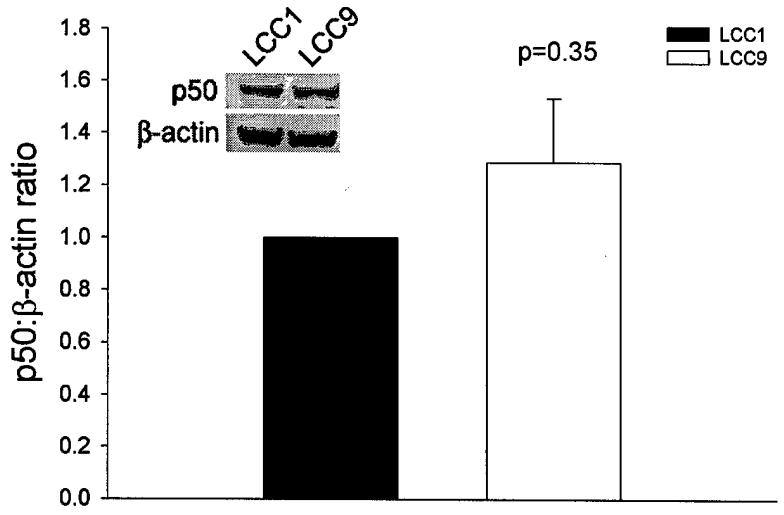
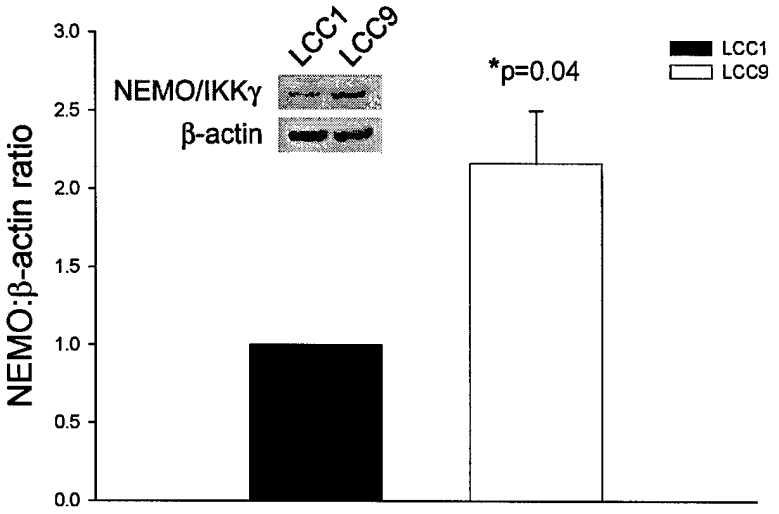


Figure 1C

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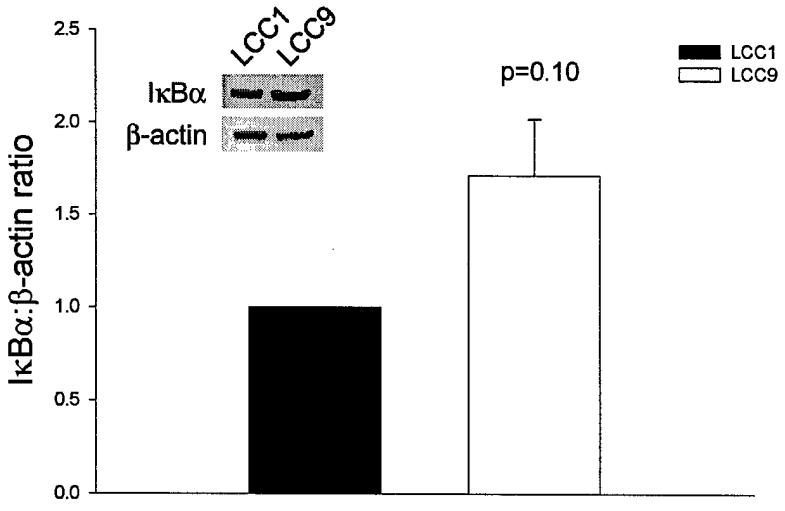

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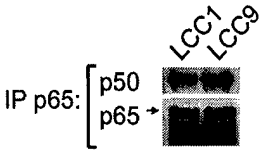


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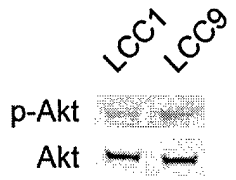


Figure 2A

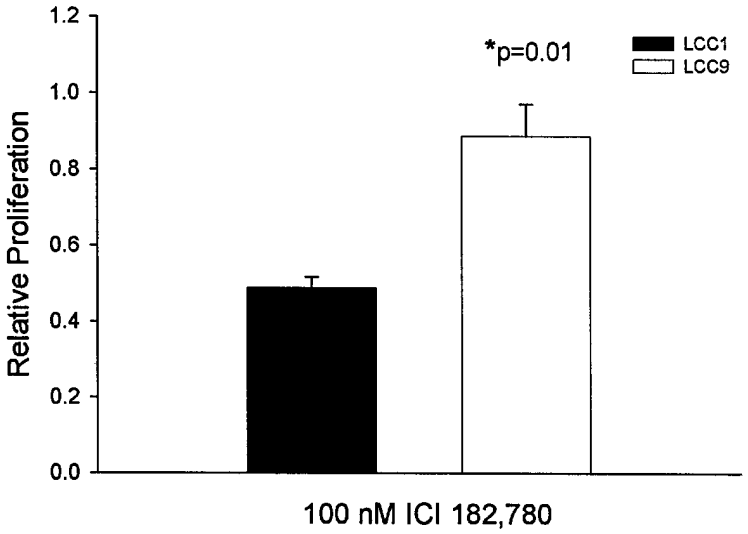



Figure 2B

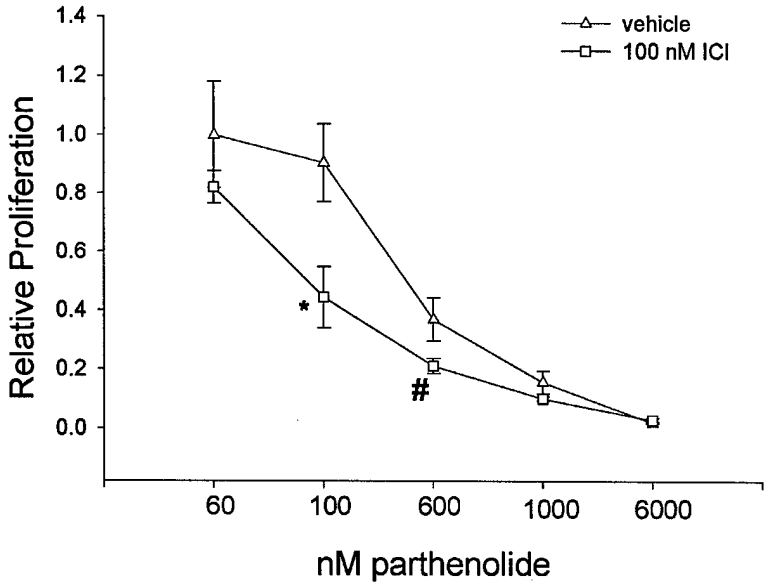
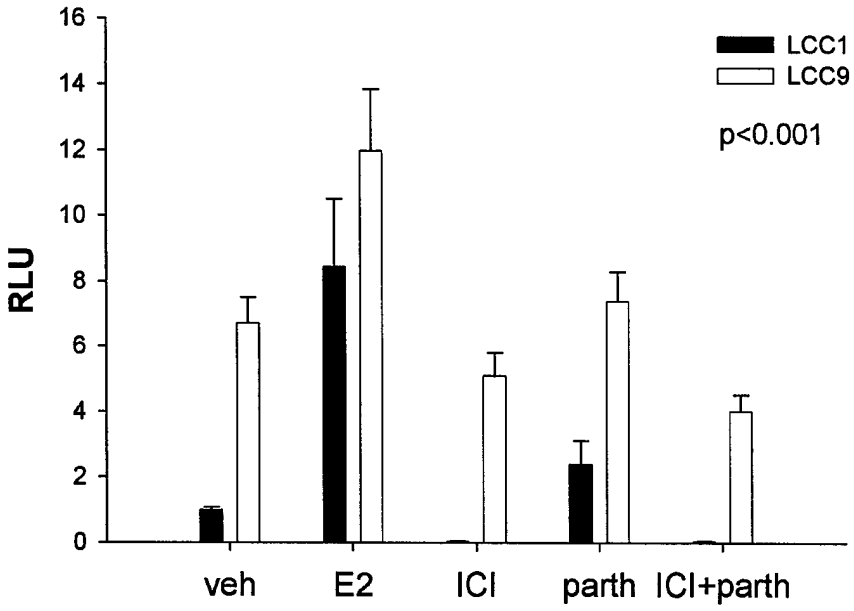


Figure 3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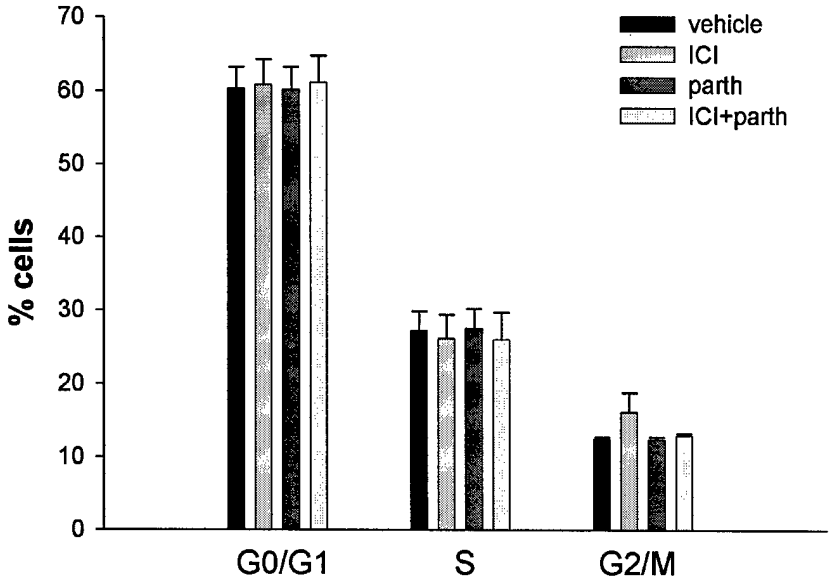
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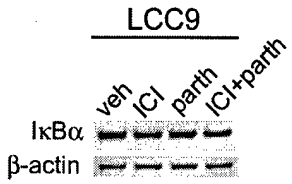

# ANTIESTROGENS, AROMATASE INHIBITORS, AND APOPTOSIS IN BREAST CANCER

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## Abstract

Antiestrogens have been the therapeutic agents of choice for breast cancer patients whose tumors express estrogen receptors (ER), regardless of menopausal status. Unfortunately, many patients will eventually develop resistance to these drugs. Antiestrogens primarily act by preventing endogenous estrogen from activating ER and promoting cell growth, which can ultimately lead to tumor cell death. Understanding the mechanism(s) by which antiestrogens cause cell death or apoptosis will be critical in our efforts to develop ways to circumvent resistance. This article focuses on antiestrogen-induced apoptosis both *in vitro* and *in vivo*. We review the clinical utility of both antiestrogens and aromatase inhibitors, and their apoptogenic mechanisms in cell culture models. Among the key signaling components discussed are the roles of Bcl-2 family members, several cytokines and their receptors, p53, NFκB, IRF-1, PI3K/Akt, and specific caspases. Finally we discuss the evidence supporting a role for apoptotic defects in acquired and *de novo* antiestrogen resistance.

## I. Introduction

Breast cancer will affect 1 in 8 women in the US this year, making it the second-most common cause of cancer-related death in women (Jemal et al., 2004). Significant progress has been made in our ability to treat and manage this disease, with both local and systemic therapies associated with an overall survival benefit in some women (EBCTCG, 1992; EBCTCG, 1998a). One of the most notable advances has been the development of targeted therapies that inhibit estrogen action, a major proliferative stimulus in the breast (Hilakivi-Clarke et al., 2002). Indeed, estrogen present within breast tumors is the most biologically active (17β-estradiol), and the average concentration (approximately 1.2 nM) should be sufficient to occupy all ERs in a breast tumor if biologically available for receptor binding (Clarke et al., 2001).

While ovariectomy has been used to treat premenopausal women for over one-hundred years (Beatson, 1896), for the past several decades antiestrogens have been the drug of choice for all patients - irrespective of menopausal status - whose tumors express estrogen receptors (ER). Antiestrogens primarily act by preventing endogenous estrogen from activating ER and promoting cell growth. Currently, the most widely used antiestrogen is Tamoxifen (TAM), which is generally well tolerated and effective in approximately 50% of all ER-positive breast cancers (EBCTCG, 1998b; EBCTCG, 1992). However, the remaining 50% of ER-positive breast cancer patients do not respond to TAM and many that show an initial response will later develop resistance (Clarke et al., 2001).

More recently, evidence has emerged to suggest that third generation aromatase inhibitors, which block estrogen biosynthesis, may be as effective as TAM in treating some postmenopausal women (Miller, 2004; Dixon et al., 2003). Whether aromatase inhibitors or the newest generation of antiestrogens such as Faslodex (ICI 182,780, Fulvestrant) will replace TAM as the first line endocrine therapy of choice remains to be seen. Nonetheless, it is already clear that various patterns of both cross-resistance and cross-sensitivity among specific antiestrogens and aromatase inhibitors exist in breast tumors. Even in cases where there is initial cross-sensitivity, for example, where response and then failure to an antiestrogen is followed by response to a second line aromatase inhibitor, the overall response rates and duration of responses is frequently lower when the aromatase inhibitor is given in the second line than when administered as a first line agent. Thus, there are very likely to be mechanisms of action and resistance that are common to both antiestrogens and aromatase inhibitors. This review will

examine the molecular mechanisms of endocrine therapy, focusing primarily on antiestrogens and on the changes that occur in programmed cell death (apoptosis).

## II. Estrogen and Estrogen Receptors

Estrogen receptors belong to a large nuclear receptor superfamily that exerts its effects by regulating the transcription of target genes (Mangelsdorf et al., 1995). Two mammalian ERs are known: ERα and the more recently identified ERβ, which share a similar domain structure with a central DNA-binding region flanked by activation function 1 and 2 (AF-1 and AF-2) domains. The carboxy-terminal AF-2 is dependent on ligand stimulation; binding of estrogen induces distinct conformational changes that allow ER to bind DNA at consensus estrogen response elements (EREs), subsequently turning on estrogen-dependent gene transcription and cell proliferation. Estrogen binding also allows the recruitment of ER coactivators that subsequently attract histone acetyltransferases, a class of chromatin remodeling enzymes that allows transcriptional activation to proceed (Hall et al., 2001). Ligand-occupied ER can function as a coregulator protein by interacting with the transcriptional machinery at binding sites for other transcription factors such as SP-1, AP-1, or NFκB (Kushner et al., 2000; McDonnell et al., 2002). Ligands other than estrogen (including antiestrogens, see below) can result in alternative receptor conformations, changes in coactivator and/or corepressor recruitment, and differential gene transcription at EREs and other sites.

In contrast, the amino-terminal AF-1 is ligand-independent and typically regulated by growth factor signaling. Epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) are two of several growth factors that have been shown to regulate ER-dependent transcription independent of estrogen stimulation, most likely through the induction of p44/42 extracellular signal regulated kinase/mitogen-activated protein kinase (Erk/MAPK) that can phosphorylate ERα on serine residue 118 within the AF-1 domain (Kato et al., 1995; Bunone et al., 1996). Another serine/threonine kinase (protein kinase A, or PKA) can be activated by cyclic AMP (cAMP) and phosphorylate ERα on Ser236 (Aronica and Katzenellenbogen, 1993; Cho et al., 1994). Phosphorylation of Ser118 and Ser236 by MAPK, PKA, or other kinases has been demonstrated to increase transcriptional activity of ERE-containing reporter genes. Although the precise contribution of ER phosphorylation events to breast tumorigenesis and the regulation of

ER activity *in vivo* are still a subject of debate (Atanaskova et al., 2002; Lannigan, 2003; Murphy et al., 2004; Joel et al., 1998), it is clear that growth factor signaling is a key component of breast cancer. Clarifying the crosstalk that occurs between growth factor and estrogen receptors will be an important step toward understanding breast cancer biology and improved management of this disease.

Another significant gap in our knowledge of breast cancer etiology is the true role of ERβ (Speirs et al., 2004; Speirs, 2002). While structurally similar to ERα in the DNA-binding and ligand-binding domains, ERβ appears to lack AF-1 function and exhibits activities distinct from those of ERα (Kuiper et al., 1996; Mosselman et al., 1996; Hayashi et al., 2003). Notably, when the two receptors are expressed together in breast cancer cells, ERβ inhibits ERα transcriptional activity (Hall and McDonnell, 1999), and in human breast tumors there appears to be an inverse correlation between ERα and ERβ expression (Bieche et al., 2001). Furthermore, stable expression of ERβ in the T47D breast cancer cell line results in the suppression of estrogen-induced cell growth (Strom et al., 2004). It has been suggested that differential recruitment of coregulatory molecules is one mechanism by which ERα and ERβ function differently in breast tissues (Muramatsu and Inoue, 2000). Another possibility is extra-nuclear distribution of ERβ, which has recently been localized to the mitochondria in cells of neuronal and cardiac origin (Yang et al., 2004).

Accumulating evidence suggests that ERα can participate in novel, transcription-independent signaling pathways outside the nucleus (Falkenstein et al., 2000). Some groups have observed ERα localized at the plasma membrane, specifically in association with lipid microdomains containing the scaffolding protein caveolin-1 (Razandi et al., 2002; Chambliss et al., 2000; Kim et al., 1999), while others have noted an association with G protein-coupled receptors (GPCRs), the nonreceptor protein tyrosine kinase (PTK) c-Src, or members of the MAPK pathway such as B-Raf and Ras (Migliaccio et al., 1996; Migliaccio et al., 2000; Razandi et al., 1999; Singh et al., 1999; Wyckoff et al., 2001). The potential functions of ER outside the nuclear environment are not clear. A report by Razandi *et al.* (Razandi et al., 2003) showed that these plasma membrane-associated receptors can interact with signaling pathways involving epidermal growth factor receptor (EGFR) and MAPK. GPCRs appear to be activated in response to estrogen, leading to the activation of Src, several matrix metalloproteases, and the release of surface-bound EGF from MCF-7 breast cancer cells. Other data support the idea that cytoplasmic

ERα links to the MAPK pathway; estrogen treatment of MCF-7 cells results in phosphorylation of the adapter protein Shc, which stimulates Erk via the activation of Grb2, Sos, and Ras (Song et al., 2002). MAPK-mediated transcriptional activation can also lead to cell growth (Roovers and Assoian, 2000). Song *et al.* (Song et al., 2004) have recently shown that the IGF-1R also plays a critical role in the rapid recruitment of ERα to the plasma membrane following E2 stimulation. Therefore, the actions of ERα through both transcriptional and non-transcriptional means appear to be critical for the control of cell proliferation, and deregulation of any one of these pathways could contribute to the aberrant cell growth seen in breast cancer.

## III. Antiestrogens

The primary mechanism of action of an antiestrogen is competition with estrogen for binding to the ER. Since the first report that ovariectomy led to a reduction in breast tumor mass in premenopausal women (Beatson, 1896), endocrine manipulation and/or antiestrogen-mediated inhibition of breast cancer cell growth have been some of the most successful targeted approaches in the treatment of estrogen-dependent breast tumors (Clarke et al., 2001; Clarke et al., 2003). The most common antiestrogen is the nonsteroidal triphenylethylene tamoxifen (TAM), an ER partial antagonist that exhibits tissue selectivity for its antagonist and antagonist activities (Clarke et al., 2001).

The most potent metabolite of TAM is 4-hydroxytamoxifen (OH-TAM), which binds to the ligand-binding region of ERα with high affinity. In bone, uterine, and cardiovascular tissues, TAM functions as a positive regulator of ER function. However, TAM induces a different conformational change than that of estrogen-bound ER; the coactivator recognition groove of ER is blocked when the receptor is bound to TAM (Shiau et al., 1999), and it has been shown that the corepressor N-CoR binds to ER occupied by TAM (Jackson et al., 1997). In breast cancer cell lines, this can result in the inhibition of estrogen-responsive gene expression and cell growth, as well as the stimulation of apoptosis or programmed cell death (Jordan, 1990; Musgrove et al., 1993). Other nonsteroidal antiestrogens such as raloxifene behave similarly to TAM both *in vitro* and *in vivo*.

The steroidal or "pure" antiestrogen Faslodex (ICI 182,780; Fulvestrant) inhibits estrogen-dependent events through a different mechanism (Howell, 2001). Faslodex is a full antagonist and enhances the ubiquitin-mediated degradation of ERα (Dauvois et al., 1992;

Nawaz et al., 1999). Interest in Faslodex as a first-line endocrine therapy is increasing. For example, Faslodex has shown significant activity as a second-line agent in women who developed TAM resistance following an initial response, and Faslodex is at least as active as the aromatase inhibitor anastrazole in inducing an objective response while enhancing overall patient survival (Howell et al., 1995; Howell and Dowsett, 1997; Howell et al., 2002; Osborne et al., 2002).

Regardless of mechanism, the ultimate goal of endocrine or antiestrogen therapy is to induce breast tumor regression. This can occur either by the inhibition of cell growth (cytostasis) or the active induction of apoptosis (cytotoxicity), and both cytostatic and cytotoxic effects of antiestrogens are observed in human tumors. The dysregulation of several molecular mechanisms and signal transduction pathways may contribute to the antiestrogen resistance phenotype. We have begun to establish several components of a broader signaling network associated with antiestrogen action and resistance (Clarke et al., 2003). However, a comprehensive assessment is outside the scope of this review and we will focus on the regulation of apoptosis in response to antiestrogens.

## IV. Aromatase Inhibitors, Estrogen Independence, and Antiestrogens

Aromatase inhibitors have been available for clinical use for several decades. Aminoglutethimide was the first such agent used in the management of invasive breast cancer, with overall response rates that are broadly comparable to those associated with ovariectomy, the progestins, and TAM (Smith et al., 1981; EBCTCG, 1998b). However, the nonselective nature of aminoglutethimide led to substantial toxicity and thus its positioning as second line treatment upon metastatic disease progression on TAM. Second and third generation aromatase inhibitors have since been developed, which have greater specificity for the aromatase enzyme and thus a more favorable safety and toxicity profile. Miller (Miller, 1997) has separated the newer generation aromatase inhibitors into two classes; those which are steroidal and compete for substrate binding (Type 1; examples are formestane, exemestane) and those which are nonsteroidal (Type II; examples are fadrozole, anastrazole, letrozole). Both type 1 and type 2 agents have comparable activity to TAM in various treatment settings, but letrozole appears to most effectively decrease circulating estrogen levels by inhibiting activity by almost 99% *in vivo* (Miller, 2004).

In premenopausal women, the primary site of estrogen biosynthesis is the ovary; in postmenopausal women adipose tissue is the most active source of aromatase activity, although, muscle mass may be a greater overall contributor to plasma estrogen levels (Miller, 2004). Antiestrogens function at the level of the estrogen receptor and thus exhibit equivalent efficacy irrespective of menopausal status. The selective aromatase inhibitors function at the level of the aromatase enzyme, such that their efficacy depends on the cessation of ovarian function either biologically (*i.e.*, by natural menopause) or medically (*i.e.*, by elective ovariectomy or the use of LHRH agonists). In some women, peripheral aromatization of serum androgens can account for up to 50% of circulating estrogens (Kirschner et al., 1982). Most serum estrogens in postmenopausal women are present as the sulfated metabolite and as such are biologically inactive. Since estrogen sulfotransferases that sulfate estrogens also are detected in breast tumors (Adams et al., 1979), the presence of biologically active estrogens further requires activity of the steroid sulfatase (STS gene; chromosome Xp22.32; EC 3.1.6.2.) (Entrez Gene, 2004b). Indeed, we have shown that expression of STS is sufficient to support the growth of estrogen dependent breast tumors (James et al., 2001).

The activity of aromatase inhibitors is not surprising, given the estrogen dependence of many breast tumors, the association of increased serum estrogen concentrations with breast cancer risk, and the high concentrations of estradiol present in tumors in postmenopausal women (Clarke et al., 2001). The target of these drugs is the aromatase enzyme (CYP19A1 gene; chromosome 15q 21.1; EC 1.14.14.1), which is part of the cytochrome P450 complex (Entrez Gene, 2004a). Molecular oxygen and NADPH are used by the enzyme to perform three hydroxylations that convert C19 steroids (androgens), usually androstenedione but also testosterone, to C18 steroids (estrogens). When testosterone is the substrate for aromatase, the product is estradiol. If the substrate is androstenedione, the product is estrone, a steroid with a relative binding affinity for ER approximately 60% that of estradiol (Kuiper et al., 1997). The final conversion of estrone to estradiol, the primary estrogen present in breast tumors, is catalyzed by the 17β-hydroxysteroid dehydrogenase type 1 enzyme (HSD17B1 gene; chromosome 17q11-q21; EC 1.1.1.62) (Ensemble, 2004). HSD17B1 is readily detected, and occasionally amplified, in breast tumors (Gunnarsson et al., 2003). Many breast tumors and adipose tissue within the breast express both aromatase (Goss et al., 2003; Miller, 2004) and HSD17B1 (Gunnarsson et al., 2003), which almost certainly contributes to the high intratumoral

concentrations of estradiol in many breast tumors (Clarke et al., 2001). Inhibitors of the HSD17B1 and/or STS enzymes may have significant clinical activity as either single agents, in combination with antiestrogens, and/or in combination with aromatase inhibitors.

Paradoxically, the long term use of some of the most effective aromatase inhibitors can induce aromatase such that there is sufficient estrogen biosynthesis to overcome the effects of the drug (Goss et al., 2003). Other potential mechanisms of resistance to the aromatase inhibitors include mutations in the aromatase gene and excessive exposure to exogenous estrogens (Miller, 2004). Estrogen hypersensitivity also may contribute to resistance to aromatase inhibitors (Santen et al., 2001), particularly if aromatase expression is induced. However, it seems unlikely that hypersensitivity or increased estradiol production would be adequate to overcome TAM activity because of the excess of antiestrogenic metabolites present in most tumors treated with this drug. Using intratumoral TAM and TAM metabolite levels rather than serum concentrations, which may not fully reflect tissue exposures, we have estimated that antiestrogenicity exceeds estrogenicity by at least two orders of magnitude in many breast tumors (Clarke et al., 2003). Other resistance mechanisms are clearly shared by antiestrogens and aromatase inhibitors, most notably the lack of ER expression.

Since both antiestrogens and aromatase inhibitors can interfere with the activation of ER by estradiol, it might be expected that similar mechanisms of sensitivity and resistance would exist. Nonetheless, many investigators use the term estrogen (or hormone) independence to be synonymous with TAM (antiestrogen) resistance. It is clear from studies in experimental models that these are very different phenotypes. Human breast cancer cell lines selected for their ability to grow in the absence of estrogens frequently retain expression of functional ER and sensitivity to antiestrogens (Katzenellenbogen et al., 1987; Clarke et al., 1989). These observations directly reflect the clinical situation in which patients with hormone receptor-positive tumors respond to an antiestrogen as first line therapy and then to an aromatase inhibitor as second line therapy (Rose, 2003). Evidence also suggests that in tumors the converse is true (Smith et al., 1981).

These experimental and clinical phenotypes clearly establish that estrogen/hormone independence is a phenotype that can be fully separated from antiestrogen resistance. Resistance to aromatase inhibitors and antiestrogens can also be separated; for example, erbB2 overexpression has been associated with a decreased likelihood of response to TAM but it has no compelling impact on the prediction of responsiveness to aromatase inhibitors (Ellis et al., 2001);

there also is clear evidence of crossresistance, as is seen with ER negativity. While estrogen/hormone independence may be more correctly applied to tumors resistant to aromatase inhibitors, it may be more correct, and more useful, to define resistance phenotypes more specifically. We have previously identified four endocrine phenotypes that reflect the diversity of responsiveness patterns and suggest applying the term multihormone resistant to tumors that are crossresistant to both antiestrogens and aromatase inhibitors (Clarke and Brünner, 1995; Clarke et al., 2003).

## V. Antiestrogens *versus* aromatase inhibitors as endocrine therapies

Endocrine therapy has established utility in treating hormone receptor-positive invasive breast cancer as well as in the setting of chemoprevention. In terms of existing disease, TAM has been the gold standard of therapy for over thirty years. Nonetheless the activity and favorable toxicity profile of the third generation aromatase inhibitors as used in treating metastatic disease have led to clinical investigations into their efficacy in early stage disease and chemoprevention. Large, randomized clinical trials have looked at anastrazole (Baum et al., 2002), (Baum et al., 2003), letrozole (Goss et al., 2003), and exemestane (Coombes et al., 2004) in place of, or in sequence with, TAM as adjuvant therapy. Early results from each of these trials suggest a survival benefit from the use or addition of aromatase inhibitors, although longer follow-up is needed to demonstrate durability of benefit and better characterize the long-term toxicities associated with these newer agents. In addition, TAM is an accepted means of prevention in women at high risk for developing breast cancer (Fisher et al., 1998) as well as in women with a personal history of ductal carcinoma *in situ* (Fisher et al., 1999). The use of other agents, such as raloxifene and various aromatase inhibitors, is recommended only in the context of clinical trials (Chlebowski et al., 2002), (Leonard and Swain, 2004). However, the three large randomized clinical trials of adjuvant endocrine therapy have demonstrated a significant reduction in the development of contralateral breast cancer, suggesting a putative and encouraging chemopreventive effect (Baum et al., 2002; Baum et al., 2003; Coombes et al., 2004; Goss et al., 2003).

Anastrozole, letrozole, and exemestane have at least comparable activity to TAM as first line endocrine therapy in the metastatic setting (Bonneterre et al., 2000; Mouridsen et al., 2001). The preferential use of these agents is increasing because of data suggestive of higher objective

response rates and improved disease-free survival, as well as concerns regarding the risk of venous thromboembolic disease and other toxicities uniquely associated with TAM. In these cases, TAM is therefore administered as a second line therapy. There is also increasing data to support the use of aromatase inhibitors in the preoperative setting, which may lead to increased opportunities for breast conserving local therapy, as well as suitable treatment alternatives who are not candidates for surgery and/or chemotherapy (Dixon & Miller, 2003; (Ellis, 2000; Dixon, 2004).

The role of the steroidal and nonsteroidal aromatase inhibitors in relation to such new "pure" antiestrogens as Faslodex is less clear. There is limited evidence of cross-resistance with TAM, and the response rates in studies of Faslodex after progression on TAM have led to its approval by the FDA for use in the treatment of metastatic breast cancer after disease progression on TAM (Howell et al., 1995; Howell, 2001). Other investigations comparing Faslodex and anastrazole as second line endocrine therapy for advanced disease suggest equivalence with a slightly more favorable side effect profile (Howell et al., 2002; Osborne et al., 2002; Robertson et al., 2003), and studies comparing Faslodex with exemestane are ongoing. The intramuscular route of administration for Faslodex – as opposed to the oral administration of these other agents – may be favorable in the context of assuring patient compliance, but issues regarding injection site discomfort and bleeding need to be addressed as well.

Standard recommendations regarding the clinical roles of steroidal and nonsteroidal aromatase inhibitors, the selective estrogen receptor modulators, and the pure antiestrogens will likely change over the next several years. Improved knowledge about the underlying mechanisms of endocrine therapy responsiveness and resistance will be instrumental toward this end, particularly with the anticipated ability to predict which subset of patients will benefit most from a given therapeutic intervention. For example, an aromatase inhibitor may be the endocrine treatment of choice in the small proportion of ER+ tumors that overexpress erbB2, irrespective of disease stage at diagnosis (Ellis et al., 2001). A similar preference may be established for breast tumors that activate similar or interrelated signaling pathways, such as EGF-receptor overexpression. The ability to combine erbB2 or EGF-receptor inhibitors with antiestrogens, perhaps upon failure on an aromatase inhibitor, offer further intriguing opportunities for future study.

Results from combining antiestrogens and aromatase inhibitors have been largely

disappointing. A major synergistic interaction may be unlikely, given the potency of each class of drug alone and their common targeting of ER mediated events. Studies combining aminoglutethimide with TAM have not shown any major advantage compared with either drug alone. Studies with the more recent aromatase inhibitors have been even less supportive of such drug combinations. Pharmacokinetic interactions between TAM and arimidex and letrozole result in poorer responses in the combination arm compared with the aromatase inhibitor alone (Dowsett et al., 1999; Dowsett et al., 2001b). Nonetheless, it remains possible that the correct combination of antiestrogen and aromatase inhibitor administered in the appropriate schedule could still prove better than either drug alone. For example, while the agonist activities of TAM are often best exhibited in the absence of estrogen (Clarke et al., 2001), the ability of Faslodex to induce ER degradation (Dauvois et al., 1992) could overcome estrogen hypersensitivity or increased aromatase expression resulting from long term exposure to an aromatase inhibitor.

## VI. Apoptosis

Apoptosis is a complex and highly regulated cellular process driven by biochemical and morphological changes that ultimately lead to DNA fragmentation and cell death. Regulation of apoptosis is essential throughout the life of all organisms, which are constantly striving for a balance between cell proliferation and cell death. Dysregulation of apoptosis can shift this balance in favor of aberrant cell growth, a hallmark of cancer. Two major signaling pathways lead to apoptosis: the cell surface receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway (Delhalle et al., 2003; Hersey and Zhang, 2003). While these processes are initiated by different means they are not mutually exclusive; both require the activation of caspases, a family of cysteine proteases that cleave their target proteins at specific peptide residues.

The extrinisic pathway is initiated in response to extracellular signals. Proapoptotic ligands, such as tumor necrosis factor-α (TNFα), TNF-related apoptosis-inducing ligand (TRAIL), or Fas-ligand (FasL), bind to their cognate receptors and can induce multimerization (Locksley et al., 2001). Activated TNF, TRAIL, or Fas receptors subsequently recruit adapter proteins to their intracellular death domains, which in turn recruit and assist in the activation of initiator caspases 8 and 10. The intrinsic pathway is more often induced in response to intracellular stimuli, such as DNA damage. Subsequently, pro-apoptotic members of the Bcl-2 family of proteins are relocalized to the mitochondrial outer membrane. Mitochondrial

membrane permeability is then compromised, leading to the release of cytochrome c that binds to the apoptotic protease-activating factor (Apaf-1) and serves to activate initiator caspase 9. Both the intrinsic and extrinsic apoptotic pathways result in the activation of effector caspases 3 and/or 7 (Strasser et al., 2000; Earnshaw, 1999).

## A. Evidence for Antiestrogen-Induced Apoptosis *In Vivo*

Antiestrogens have the ability to elicit cell cycle arrest and/or apoptosis. In the past several years, a great deal of progress has been made in understanding the apoptotic pathways utilized by breast cancer cells and how antiestrogens impinge on this process (Mandlekar and Kong, 2001). From a clinical perspective, the induction of apoptosis is an important component of breast cancer regression. If antiestrogens solely arrested tumor cells in the $G_0$ phase, it is likely that some cells would eventually escape this inhibition and resume proliferation. In addition, cells within the tumor mass would not be actively eliminated, beyond the turnover normally expected, and improvements in survival might not be expected. However, there is clear evidence that antiestrogen therapy reduces breast tumor size and increases overall survival (EBCTCG, 1998b; EBCTCG, 1992), indicating that apoptosis is a key feature of these drugs' activity *in vivo*. Both TAM and Faslodex are capable of inducing apoptosis; Raloxifene may be less effective (Dowsett et al., 2001a; Ellis et al., 1997).

## B. Apoptosis Pathways in Breast Cancer Model Systems

Much of what we know about the mechanism(s) of antiestrogen-induced apoptosis comes from the study of a few breast cancer cell line model systems (Table 1). Both the MCF-7 and T47D cell lines were derived from metastatic pleural effusions of invasive ductal carcinoma (Clarke et al., 2001). Another ER-positive and hormone-dependent cell line arising from an invasive ductal carcinoma metastasis is ZR-75-1. Since it is from these three lines that the majority of antiestrogen resistance models have been generated (Clarke et al., 2001), it is important to understand the major classical apoptotic pathways that are affected by estrogen, estrogen withdrawal, and antiestrogen treatment of the sensitive (parental) cells.

### 1. Cell Surface Receptors:

The TNF, TRAIL, and FasL receptors are three key mediators of the extrinsic apoptotic pathway, and their ligand-dependent activation generally results in rapid cell death (Locksley et al., 2001). Expression and function of these receptors has been described in the three most widely used models of estrogen-dependent breast cancer, the human cell lines MCF-7, T47D and ZR-75-1. In MCF-7 cells, TNF stimulation leads to apoptosis that proceeds via the cleavage of Bak, a Bcl-2 family member (Suyama et al., 2002); various clones of MCF-7 cells exhibit different degrees of response to TNF-mediated apoptosis (Burow et al., 1998). In ZR-75-1 cells, TNF inhibits growth of hormone-dependent cell lines by inducing cell death that is potentially mediated by changes in c-myc expression (Mueller et al., 1996). However, not all estrogen-dependent cells respond to TNF by inducing apoptosis; TNFR activation in T47D cells leads instead to cell cycle arrest at the $G_1$/S checkpoint (Pusztai et al., 1993).

Breast cancer cell lines also exhibit divergent responses to FasL. T47D cells exhibit surface expression of both FasL and the Fas receptor, and stimulation with exogenous FasL leads to apoptosis (Ragnarsson et al., 2000; Keane et al., 1996). ZR-75-1 cells also undergo Fas-dependent cell killing when treated with the CH-11 activating antibody (Tong et al., 2001). While MCF-7 cells also express FasL (Gutierrez et al., 1999), there are conflicting reports as to whether these cells are sensitive or resistant to Fas-dependent apoptosis. Mullauer *et al.* (Mullauer et al., 2000) report that MCF-7 cells are not sensitive to Fas activation by CH-11 despite their high levels of FasL expression. In contrast, the FasL present in conditioned media from normal mammary epithelial cells is reported to induce death in MCF-7 cells (Toillon et al., 2002).

Apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) occurs primarily in cancer cells, while normal cells are relatively unaffected. Thus, the TRAIL pathway is a target of significant interest for the development of new cancer therapies (Fulda and Debatin, 2004). While parental MCF-7, T47D, and ZR-75-1 cells are all resistant to TRAIL-mediated cell killing (Keane et al., 1999), sensitivity to TRAIL can be restored. For example, one consequence of TRAIL treatment is activation of the pro-survival nuclear factor kappa B (NFκB) transcription factor, and inhibition of NFκB can restore TRAIL-induced cell death (Nakshatri et al., 2004). Overexpression of interferon gamma (IFNγ) can also enhance sensitivity to TRAIL-dependent apoptosis in MCF-7 cells (Ruiz et al., 2004), probably by activating the transcription factor and tumor suppressor interferon regulatory factor-1 (IRF-1) (Clarke et al., 2004). Involvement of

IRF-1 is of particular interest because we have recently shown that IRF-1 expression is down-regulated in antiestrogen-resistant MCF-7/LCC9 cells (Gu et al., 2002) and that a dominant-negative IRF-1 blocks the proapoptotic effects of the steroidal antiestrogen Faslodex in MCF-7 and T47D cells (Bouker et al., 2004) (discussed below).

Exposure of MCF-7, T47D, or ZR-75-1 cells to antiestrogens has the potential to affect signaling via the TNF and Fas/FasL pathways. In T47D cells, TAM upregulates expression of surface FasL (Nagarkatti and Davis, 2003). Estradiol treatment of MCF-7 and T47D breast cancer cells also can increase FasL expression, and TAM decreases FasL expression (Mor et al., 2000). MCF-7 cells that have been subjected to long-term estrogen deprivation undergo apoptosis when treated with estradiol and this correlates with increased expression of FasL (Song and Santen, 2003). It is not clear what factors contribute to these conflicting results. FasL upregulation appears to play a major role in immune evasion. Activated T-cells expressing the Fas receptor can be killed by breast cancer cells that have upregulated FasL (Gutierrez et al., 1999) implying that FasL upregulation is not necessarily beneficial to inducing the apoptosis of breast cancer cells *in vivo*.

Estradiol treatment of MCF-7 cells can abolish TNF-mediated apoptosis via effects on downstream apoptotic mediators such as Bcl-2, and this can be reversed by exposure to Faslodex (Burow et al., 2001). Faslodex treatment enhances cell death induced by TNF under these conditions, and expression of both the TNF receptor and TNF receptor-associated death domain (TRADD) are increased by TAM or Faslodex treatment of MCF-7 cells (Smolnikar et al., 2000). Co-treatment of MCF-7 cells with TNF and TAM also increases cell death (Matsuo et al., 1992). Together these data implicate a significant degree of crosstalk between TNF- and antiestrogen-induced apoptosis.

**2. Bcl-2-related Molecules:**

Members of the Bcl-2 family of proteins perform either antiapoptotic or proapoptotic functions focused on the maintenance or disruption of mitochondrial membrane integrity (Gross et al., 1999). Family members contain one or more Bcl-2 homology (BH) domains, BH1-BH4. While the antiapoptotic Bcl-2 and Bcl-$X_L$ contain all four BH domains, the proapoptotic family members such as Bax and Bak generally lack BH4 or like Bik contain only BH3 domains. A comprehensive discussion of all twenty Bcl-2 family members is outside the focus of this review;

therefore, we will focus on those genes most strongly implicated in affecting antiestrogen responsiveness.

The pro-apoptotic Bax and Bak molecules are expressed in MCF-7, T47D, and ZR-75-1 cells (Niu et al., 2001; Tong et al., 2001; Leung et al., 1998; Mooney et al., 2002). In MCF-7 cells, Bak is cleaved and thereby activated during the process of TNF-initated apoptosis (Suyama et al., 2002). Bak expression decreases upon estradiol stimulation and increases in MCF-7 cells stably transfected with the aromatase gene when this activity is blocked by aromatase inhibitors (Leung et al., 1998; Po et al., 2002; Thiantanawat et al., 2003). However, others have reported that neither Bax nor Bak protein levels in these cell lines are affected by estrogen or antiestrogens such as TAM and Faslodex (Salami and Karami-Tehrani, 2003; Gompel et al., 2000; Kandouz et al., 1999; Zhang et al., 1999). Thus, while Bax and Bak are expressed in these model systems, there is no clear consensus on what role they may play in antiestrogen-induced apoptosis.

The BH3-only protein Bik has recently been suggested to play a critical role in the antiestrogen-induced apoptosis of breast cancer cells (Hur et al., 2004). Bik mRNA is upregulated in MCF-7, T47D, and ZR-75-1 cells following exposure to Faslodex but detectable levels of Bik protein are only observed in MCF-7. Bik could also be induced by culturing MCF-7 cells under estrogen-deprived conditions, and small inhibitory RNA (siRNA) directed against Bik effectively eliminated Faslodex-mediated apoptosis (Hur et al., 2004). Moreover, Frasor *et al.* (Frasor et al., 2003) have reported that Bik is one of several genes downregulated by estradiol and upregulated by Faslodex but not the nonsteroidal antiestrogens Raloxifene or OH-TAM, suggesting that this molecule may be one mediator of apoptosis in response to steroidal antiestrogens.

Activities of the antiapoptotic factors Bcl-2 and Bcl-$X_L$ have been widely studied in breast cancer cell lines. The major mechanism by which these molecules inhibit apoptosis is by forming dimers with proapoptotic Bcl-2 family members and preventing cytochrome c release from the mitochondria. While Bcl-$X_L$ appears to be expressed basally in all three cell lines (Simoes-Wust et al., 2000), Bcl-2 protein is undetectable in T47D cells (Elstner et al., 2002; Butt et al., 2000). However, estradiol can upregulate Bcl-2 in T47D, ZR-75-1, and MCF-7 cells (Gompel et al., 2000), and others have shown that TAM and Faslodex can decrease Bcl-2 expression (Kandouz et al., 1999; Thiantanawat et al., 2003; Burow et al., 2001; Somai et al.,

2003; Zhang et al., 1999). This correlates with the induction of apoptosis (Zhang et al., 1999) and occurs at the level of transcription, as luciferase expression driven by the Bcl-2 promoter and two consensus estrogen response elements (EREs) within the Bcl-2 coding region is increased two-fold in the presence of estradiol and inhibited upon addition of Faslodex to MCF-7 cells (Somai et al., 2003). It is also important to note that induction of apoptosis in the rat mammary gland by ICI 182,780 correlates with the downregulation of Bcl-2 (Lim et al., 2001). Hence, it would seem that Bcl-2 is an important estrogen-regulated gene in these breast cancer cells and that attenuation of this prosurvival signal is directly involved in the apoptotic response to antiestrogens.

**3. Caspases:** Regardless of the means by which apoptosis is induced, caspases are responsible for initiation of the physical changes associated with cell death. Initiator and effector caspases are synthesized in an inactive form, and when cell death signals are received these molecules are activated by proteolytic cleavage (Strasser et al., 2000; Earnshaw, 1999). The range of substrates is varied and includes proteins involved in DNA repair, cell cycle progression, and invasion or metastasis, as well as other caspases.

Caspases 3 and 7 are the two most commonly studied effector caspases. MCF-7 cells lack expression of caspase 3 due to a 47-base pair deletion that results in exon skipping, whereas ZR-75-1 and T47D cells are caspase 3-competent (Janicke et al., 1998). MCF-7 cells have clearly adapted to this deficiency by using other members of this family such as caspases 9, 7, or 6. Liang *et al.* (Liang et al., 2001) have demonstrated that MCF-7 cells treated with neocarzinostatin, a mitotic inhibitor, undergo apoptosis that depends on the activation of caspase 9, followed by 7 and 6. In contrast, MCF-7 cells exhibit caspase 6 activation (but not caspase 7 activation) in response to the protein kinase inhibitor staurosporine (Mooney et al., 2002). Thus, specific caspase activation cascades may be dependent on the apoptotic stimulus.

TAM at a dose of 5 μM has been shown to modestly increase the activity of caspases 8 and 9 in MCF-7 cells, with no effects on caspase 3-like substrates (Mandlekar et al., 2000). Salami *et al.* (Salami and Karami-Tehrani, 2003) have also reported that 1 μM TAM, while capable of inducing cell death, is not sufficient to produce cleavage of caspase 3-like substrates. However, Fattman *et al.* (Fattman et al., 1998) showed that TAM treatment could induce the cleavage of poly-(ADP-ribose) polymerase (PARP), a caspase 3/7 substrate. In response to

aromatase inhibitors, MCF-7 cells stably transfected with the aromatase gene exhibit strong activation of caspases 9, 6, and 7 accompanied by PARP cleavage, while TAM and Faslodex treatment also activate these enzymes but to a lesser degree (Thiantanawat et al., 2003). In T47D cells, TAM treatment induces caspase 3 activity (Ellis et al., 2003a). Since caspase 8 is frequently considered to be activated by extrinsic cell death signals (whereas caspase 9 participates in intrinsic apoptosis), current data suggest that antiestrogens may affect both apoptotic signaling pathways and future studies should attempt to clearly define which pathway predominates.

**4. p53:** The tumor suppressor gene p53 and its family members p63 and p73 are global regulators of cell cycle arrest and apoptosis, and p53 is inactivated in about 30% of all breast cancers. In response to DNA damage, ionizing radiation, or cytotoxic drugs, expression of p53 is stabilized and transcription of p53 target genes is increased. Target genes include those that promote either cell cycle arrest, such as the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$, or apoptosis, such as the proapoptotic Bcl-2 family member Bax. p53 has also been shown to inhibit the expression of Bcl-2 (Haldar et al., 1994) or relocalize to the mitochondria and prevent Bcl-2-or Bcl-$X_L$-mediated survival functions (Marchenko et al., 2000). Thus, in the presence of a growth-inhibitory signal, p53 may determine whether a cell initiates growth arrest or cell death.

While MCF-7 and ZR-75-1 cells contain wild type p53, T47D cells express a mutated form containing a phenylalanine substitution at codon 194 (Strano et al., 2000). This residue lies in the central core of the molecule and mutations here affect the DNA binding capacity of p53. Strano *et al.* (2000) have also shown that p53 Phe194 in T47D cells can bind to p63 or p73 and inhibit transcription of their target genes. Given its central role in tumor suppression, it might be predicted that p53 is involved in breast cancer cell responses to antiestrogens but the current data are contradictory.

Some studies have shown that estradiol treatment increases p53 expression in MCF-7 (Lilling et al., 2002; Guillot et al., 1996) and T47D cells (Dinda et al., 2002), the latter in spite of their mutant p53. Lilling *et al.* (Lilling et al., 2002) also reported that TAM and estrogen depletion reduced p53 levels, and Faslodex can reverse p53 induction by estrogen in T47D cells (Dinda et al., 2002). In transcriptional studies of the P1 promoter of p53, which was transiently expressed in MCF-7 cells, Hurd *et al.* (Hurd et al., 1999) found that estrogen and TAM but not

Faslodex could induce P1 transcriptional activity. In contrast, Fattman *et al.* (Fattman et al., 1998) observed no increase in MCF-7 cell p53 levels in response to TAM, despite a significant induction of apoptosis and retinoblastoma protein dephosphorylation. Zhang *et al.* (Zhang et al., 1999) similarly report no induction of p53 by TAM in this cell line.

In light of these conflicting results, it is also important to consider the intracellular distribution of p53 and not only its expression level. When intracellular localization was examined in MCF-7 cells, p53 was exported to the cytoplasm following estrogen exposure and Faslodex reversed this effect (Molinari et al., 2000). Interestingly, Lilling *et al.* (Lilling et al., 2002) observed that p53 is primarily cytoplasmic in the TAM resistant MCF-7/LCC2 cell line. In summary, any role for p53 in antiestrogen-induced apoptosis is complex and further investigations are needed to clarify the relative importance of expression and subcellular localization. p53's role in the apoptotic response to antiestrogens *in vivo* is also unclear. Some investigators have reported a significant correlation between p53 mutation and a poor response to TAM (Berns et al., 2000), whereas others have found no association between response and p53 expression or mutation (Askmalm et al., 2004; Berry et al., 2000).

**5. PI3K/Akt and NFκB:** Two other major contributors to prosurvival and antiapoptotic signaling in breast cancer cells are phosphatidylinositol 3-kinase (PI3K) and nuclear factor kappa B (NFκB). PI3K and its major downstream target Akt are activated following stimulation of a wide range of growth factor receptors and GPCRs, and inappropriate activation or amplification of these molecules has been linked to many cancers including those of the breast (Luo et al., 2003). Consequently, the effect of chemotherapeutics, including antiestrogens, on the PI3K/Akt axis is an important consideration. Stimulation of MCF-7 cells with estrogen rapidly induces PI3K/Akt activity through the ErbB2 receptor signaling pathway, and this signaling could be inhibited by both TAM and Faslodex (Stoica et al., 2003). Campbell *et al.* (Campbell et al., 2001) report that Akt is protective against TAM-induced apoptosis. PI3K is also important for estrogen-induced cell cycle progression of MCF-7 cells (Castoria et al., 2001), and estrogen induction of c-fos transcription is also dependent upon PI3K (Duan et al., 2002). However, in MCF-7 and ZR-75-1 cells, recent evidence suggests that PI3K activation interferes with estrogen receptor's ability to repress the transcription of genes that induce cell invasion and motility (such as interleukin-6) by reducing ER expression (Bhat-Nakshatri et al., 2004). Taken together, this

could constitute a feedback loop in which the initial estrogen stimulation is later controlled or downregulated by activated PI3K. In T47D cells, PI3K activation can inhibit cell death induced by FasL (Gibson et al., 1999).

One of the many targets of activated PI3K and Akt is NFκB, although this is only one mechanism by which NFκB can be stimulated. The NFκB family of transcription factors contains five members that form dimers and regulate the transcription of various genes including cytokines, cell adhesion molecules, the pro-proliferative proteins c-myc and cyclin D1, and several inhibitors of apoptosis (Chen and Greene, 2004). Inhibitors of the NFκB pathway show promise as anticancer agents (Epinat and Gilmore, 1999), since constitutive NFκB activity is widely observed in many tumor types (Baldwin, 2001). We and others have shown that NFκB activity increases in breast cancer cells as they acquire the ability to grow in the absence of estrogen or in the presence of antiestrogen (Nakshatri et al., 1997; Pratt et al., 2003), and that expression of the p65 RelA subunit of NFκB is increased in MCF-7/LCC9 antiestrogen-resistant cells (Gu et al., 2002). Therefore, NFκB appears to play a critical role in the cellular response to antiestrogens.

## VII. Antiestrogen Resistance and Defects in Apoptosis

Since antiestrogens such as TAM and Faslodex can induce apoptosis *in vitro* and *in vivo* (Figure 1), development of resistance to these agents may coincide with defects in cell death signaling or execution. There are two major classes of antiestrogen resistance: acquired resistance and intrinsic or *de novo* resistance. *De novo* resistance could be attributed to a lack of both ER and PR expression, but this does not account for the many ER+ and/or PR+ tumors that do not respond to antiestrogens. In those cases where resistance is acquired, ER expression is, for the most part, retained (Clarke et al., 2003). Alternative growth pathways, and coinciding defects in apoptosis, are likely to be responsible for at least some cases of acquired antiestrogen resistance.

### A. Apoptosis in Antiestrogen-Resistant Tumors

Evidence suggests a link between expression of apoptotic factors and the efficacy of endocrine therapy in patients. In some studies, it is necessary to separate those cases that recur because they have a poorer overall prognosis, rather than simply acquired resistance to the

treatment administered. Nonetheless, phosphorylated Akt, which is a key mediator of PI3K signaling, is associated with breast cancer recurrence and metastasis to distant sites (Perez-Tenorio and Stal, 2002). An association between p53 mutation and a poor response to TAM also has been reported (Berns et al., 2000). However, the role of p53 in affecting antiestrogen responsiveness is unclear. Elevated expression of wild type p53 has also been shown to be associated with a sub-optimal antiestrogen response (Daidone et al., 2000; Bottini et al., 2000). It is suggested that this is due to the association of p53 with a dedifferentiated phenotype, but given the complexity of p53 signaling there are likely to be additional explanations. For example, nuclear versus cytoplasmic localization of p53 may play a role in its ability to promote apoptosis in response to antiestrogens (Lilling et al., 2002).

The role of Bcl-2 in affecting antiestrogen responsiveness also is not clearly defined. Some groups have reported that decreased Bcl-2 is correlated with reduced TAM effectiveness (Gasparini et al., 1995; Daidone et al., 2000; Silvestrini et al., 1996), which is somewhat surprising considering the prosurvival functions of Bcl-2. In contrast, Cameron *et al.* (Cameron et al., 2000) showed that Bcl-2 levels can fall after 3 months of TAM therapy but only in those patients that respond positively to the treatment. While apoptosis levels within breast tumors are high after the first 24 hours of treatment, cell death is markedly decreased at the 3 month time point and within the residual cancer cell population there are higher levels of Bcl-2 (Ellis et al., 1998). Given these observations, other markers of apoptotic activity should be more closely studied to determine their connection to antiestrogen-induced apoptosis *in vivo*.

## B. Apoptosis in Models of Acquired Antiestrogen Resistance

We have begun to examine a network of apoptotic signaling, identified in several of human breast cancer cell lines and variants. Here, we focus on two molecules with functional interactions (IRF-1 and NFκB) that are strongly implicated as key nodes in a broader gene network associated with signaling from ER activation/inactivation to cell cycle progression and apoptosis. Despite their ability to form heterodimers and selectively regulate the transcription of genes such as iNOS (Saura et al., 1999), IRF-1 and NFκB clearly exhibit opposing activities; generally, IRF-1 is proapoptotic and NFκB is antiapoptotic.

**1. IRF-1**: The transcription factor IRF-1 is a major mediator of type I and II interferon (IFN) signaling. Activation of IFN receptors results in the activation of the Janus kinase/signal transducer and activation of transcription (Jak/Stat) pathway, which directly leads to the induction of IRF-1 expression (Kroger et al., 2002). In addition to its role in the immune response, IRF-1 also exhibits tumor suppressor activities in cancer cells; expression is frequently decreased or lost in tumors and cell lines of hematopoetic origin (Sillman et al., 1993). IRF-1 knock-out mice exhibit greatly increased tumor formation induced by the oncogenes myc and ras (Nozawa et al., 1999), two oncogenes implicated in breast cancer. One immunohistochemical study (Doherty et al., 2001) has also shown that IRF-1 expression is decreased in neoplastic versus normal human mammary tissue, suggesting this molecule may be involved in breast tumorigenesis.

We first reported that expression of IRF-1 is downregulated in the MCF7/LCC9 cells (Gu et al., 2002). This cell line is estrogen-independent and, while it was selected for resistance to the steroidal antiestrogen Faslodex 182,780, MCF7/LCC9 cells are also crossresistant to OH-TAM (Brünner et al., 1997). We have now shown that IRF-1 plays an important functional role in the apoptotic response to the antiestrogen Faslodex (Bouker et al., 2004). IRF-1 mRNA levels and transcriptional activity are significantly repressed in the MCF7/LCC9 cell line as compared to MCF-7 cells. Furthermore, while expression of IRF-1 is induced by Faslodex in antiestrogen-sensitive (MCF-7) cells, this regulation is lost in the resistant MCF7/LCC9 cells. Expression of IRF-1 in antiestrogen-resistant MCF7/LCC9 cells could be rescued by treating cells with the cytotoxic drug adriamycin, indicating that global transcriptional regulation of IRF-1 is not defective. To address the mechanism of action of IRF-1 in antiestrogen response we generated MCF-7 and T47D cells that stably overexpress a dominant negative IRF-1 (dnIRF-1) (Bouker et al., 2004). The dnIRF-1 construct lacks the carboxyl-terminal transcriptional activation domain, successfully inhibits IFNγ-stimulated transcription, and significantly reduces antiestrogen sensitivity in both cell lines. Importantly this occurs via a reduction in Faslodex-induced apoptosis but not cell cycle arrest, strongly linking IRF-1 function to the apoptotic action of this antiestrogen (Bouker et al., 2004).

IRF-1 is known to play a role in cell death in response to other cytotoxic agents. IFNγ can induce cell death in MCF-7 cells, and IRF-1 has been shown to contribute to apoptosis via enhanced expression and activity of caspase 8 (Ruiz-Ruiz et al., 2004). IFN-mediated

sensitization of MCF-7 cells to apoptosis induced by TRAIL correlates with increased activity of IRF-1 (Clarke et al., 2004). IRF-1 is also known to cooperate with the tumor suppressor p53 (Tanaka et al., 1996). As discussed above, MCF-7 and MCF7/LCC9 cells express normal p53 while T47D cells contain mutated p53. Since dnIRF-1 abrogates the apoptotic response to antiestrogens in both the MCF-7 and T47D cell lines, either IRF-1 is functioning independently of p53, or IRF-1-dependent apoptosis in MCF7 and T47D cells is regulated by different signaling pathways. We are currently examining in detail these and other molecular features of antiestrogen- and dnIRF-1-regulated apoptosis in several antiestrogen resistant model systems.

**2. NFκB**: NFκB is one of many targets downstream of the PI3K/Akt pathway and has been linked to the development of estrogen independence and antiestrogen resistance in breast cancer cells (Nakshatri et al., 1997; Pratt et al., 2003). In performing expression analysis of the antiestrogen-resistant MCF-7/LCC9 cells, we determined that expression of the p65 RelA subunit of NFκB is increased two-fold while NFκB-dependent transcriptional activity is increased up to 10-fold in the resistant cells (Gu et al., 2002). Furthermore, MCF7/LCC9 cells are selectively sensitive to growth inhibition by parthenolide, a small molecule inhibitor of NFκB. This led us to hypothesize that NFκB is a key effector of antiestrogen-induced apoptosis, and that the MCF7/LCC9 cells may be more dependent on NFκB-mediated signaling following the acquisition of resistance.

Subsequent studies have supported this hypothesis. Upstream of NFκB, the inhibitor of kappa B kinase (IKK) complex phosphorylates the inhibitor IκB, allowing for the release of the NFκB dimer and its transition to the nucleus (Chen and Greene, 2004). The IKK complex is comprised of 2 catalytic subunits (α and β) and a regulatory subunit known as IKKγ, or NEMO. Further analysis of the NFκB pathway in the MCF7/LCC9 cells revealed that expression of NEMO is also significantly increased when compared with the antiestrogen-sensitive MCF7/LCC1 cells, which may partially explain the observed increase in NFκB transcriptional activity (Riggins *et al.*, submitted). In further studies with parthenolide we confirmed that MCF7/LCC9 cells were sensitive to this inhibitor and while MCF7/LCC9 cells do not respond to Faslodex, the combination of parthenolide and Faslodex results in a synergistic 4-fold inhibition of cell growth. Importantly, the synergistic effect of parthenolide and Faslodex is due to a significant increase in apoptosis and has no impact on cell cycle regulation (Riggins *et al.*

submitted). Given the emerging interest in NFκB inhibitors as anticancer therapies and the fact that parthenolide has shown safety in Phase I clinical trials (Curry, III et al., 2004), we propose that further preclinical studies should rigorously investigate the combination of antiestrogens and parthenolide in the treatment of ER-positive breast cancer.

## VII. *De Novo* Antiestrogen Resistance

*De novo* or intrinsic resistance to antiestrogens could be the consequence of several different events, only one of which is development of an ER-negative tumor. Amplification or overexpression of genes that promote antiestrogen-resistant growth and/or disrupt antiestrogen-induced apoptosis could confer resistance. While a number of growth regulatory molecules have been implicated in antiestrogen response *in vitro* and *in vivo,* including EGFR, erbB2, ERβ, cyclin E and cyclin D1 (Dorssers et al., 2001), very few have independent predictive or prognostic power for determining a patient's chances of responding favorably to endocrine therapy. Two such candidates are the breast cancer antiestrogen resistance-1 and -3 (BCAR1 and BCAR3) genes.

BCAR1 and BCAR3 were discovered in an *in vitro* random insertion mutagenesis screen for antiestrogen resistance genes performed by Dorssers et al. (Dorssers et al., 1993). Estrogen-dependent ZR-75-1 cells were infected with retroviruses, selected in media containing 1 μM TAM, and resistant clones were isolated and analyzed for common integration sites. BCAR1, the first locus, was identified as the human homologue of the rat protein p130Cas (Cas) (Brinkman et al., 2000). Stable transfection of ZR-75-1 cells with BCAR1 cDNA permits the growth of these cells in the presence of either TAM or Faslodex, but not conventional cytotoxic drugs such as doxorubicin, 5-fluorouracil, or methotrexate. In a study of BCAR1 in material from 937 primary human breast tumors, almost 10% exhibited strong staining (van der et al., 2000a). These patients exhibited significantly poorer survival and increased rates of non-response to TAM. Moreover, high BCAR1/Cas expression was an independent predictor of reduced disease-free survival and non-response to TAM even when factors such as age and menopausal status were excluded.

Subsequent studies have begun to address the mechanism of BCAR1/Cas action in antiestrogen response. Van der Flier *et al.* (van der et al., 2000b) also examined tumor material from patients with acquired TAM resistance and those who had not received endocrine therapy.

They observed no significant change in BCAR1 levels between these two groups, further distinguishing BCAR1 as a predictor for *de novo* rather than acquired resistance. Immunohistochemical studies of normal and neoplastic mammary tissue revealed that BCAR1 is expressed in the luminal epithelium and vasculature, but not in the stroma or myoepithelium (van der et al., 2001). The BCAR1-positive population was also immunoreactive for ER, suggesting that the BCAR1/Cas effects on antiestrogen-resistant growth occur in the same population of cells targeted by the antiestrogens.

While the molecular mechanisms of BCAR1/Cas-mediated antiestrogen resistance are still being uncovered, a great deal is known about the function of this protein in other contexts. Cas is an adapter molecule containing multiple protein-protein interaction domains that has been implicated in such diverse cellular processes as migration and invasion, survival, proliferation, oncogenic transformation, and bacterial engulfment (Bouton et al., 2001). In the case of breast cancer and antiestrogens, interaction of Cas with the non-receptor protein tyrosine kinase c-Src may be of particular importance. Cas, which is both a substrate and an activator of Src, is tyrosine phosphorylated on multiple residues in response to stimuli that activate Src (Ojaniemi and Vuori, 1997; Casamassima and Rozengurt, 1997), and Src appears to be the preferred kinase that phosphorylates Cas (Ruest et al., 2001). Moreover, the carboxyl terminus of Cas contains binding sites for both the Src-homology 2 and 3 (SH2 and SH3) domains, allowing Src binding and conformational change leading to increased kinase activity (Burnham et al., 2000; Burnham et al., 1999). 30-70% of breast cancers have been reported to overexpress c-Src (Koster et al., 1991; Ottenhoff-Kalff et al., 1992) and these tumors' elevated kinase activity appears to derive from overexpression rather than activating mutations (Biscardi et al., 1998; Verbeek et al., 1996). Thus, Cas-mediated Src activation could play a major role in proliferation and antiestrogen resistance.

BCAR3 can also independently induce estrogen independence (Yu and Feig, 2002) and TAM or Faslodex resistance in MCF-7 and ZR-75-1 cells (van Agthoven et al., 1998). This gene is also known as NSP2 (Lu et al., 1999) and AND-34 which is the murine homologue (Cai et al., 1999). The function of BCAR3/AND-34 is less well-defined than that of BCAR1. While it has been suggested that this molecule has guanine nucleotide exchange factor activity toward several Ras family GTPases (Gotoh et al., 2000), other groups have questioned these findings (Quilliam et al., 2002; Bos et al., 2001). More recently it has been reported that AND-34 activates the Rac

GTPase as well as transcription from the cyclin D1 promoter (Cai et al., 2003). Importantly, BCAR3/AND-34 physically associates with BCAR1/Cas (Cai et al., 1999) and we have recently shown that Cas and AND-34 synergistically promote Src kinase activation and cell migration (RIggins et al., 2003). Enhancement of cell motility is dependent on Src kinase activity and coincides with a redistribution of Cas to the plasma membrane. Hence, the interaction of Cas and AND-34 has significant functional consequences for the cell, and the collaboration of these molecules in the promotion of breast cancer and antiestrogen resistance, as well as their potential suppression of apoptosis, should be further studied.

## IX. Summary and Future Directions

It is clear that many aspects of antiestrogen- and aromatase inhibitor-induced apoptosis, as well as how defects in the apoptotic pathway may be contributing to resistance, are poorly understood. Further studies in several key areas will help to increase our understanding of these problems and greatly improve the clinical management of breast cancer. One is the examination of apoptosis mechanisms in the current cell culture models of antiestrogen resistance, which should uncover important molecular targets that can be validated *in vivo*. Alongside this effort, we should also design new clinical studies that can identify biomarkers of response *vs*. non-response in breast cancer patients treated with antiestrogens and aromatase inhibitors. Ellis *et al.* (Ellis et al., 2003b) have begun a Phase II trial of the aromatase inhibitor letrozole with the goal of analyzing changes in gene expression during the first month of neoadjuvant therapy prior to surgery. Preliminary data from this study indicate dramatic changes in mediators of both apoptosis and the cell cycle, and it is hoped that the complete array of data from this and other trials will form the basis for an expression profile that will be able to accurately predict which patients will respond favorably to endocrine therapy.

Our ultimate goal is to prevent or reverse resistance to antiestrogens. With a greater understanding of the mechanisms of apoptosis that are at work in breast cancer cells, we should also be able to design combination therapies that inhibit estrogen receptor activities as well as the function of those molecules that contribute to the resistant phenotype. Awada *et al.* (Awada et al., 2003) have recently reviewed many of the new treatments being studied in metastatic breast cancer. Several of these, such as the Bcl-2-specific antisense Genasense, PI3K pathway inhibition, the proteasome inhibitor Velcade, or adenoviral delivery of wild type p53, may prove

useful in combination with TAM, Faslodex, or aromatase inhibitors. It is hoped that these and other targeted therapies will increase the clinical efficacy of antiestrogens, and greatly improve survival and quality of life for breast cancer patients.

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**Table 1.** Expression of apoptosis-related molecules in MCF-7, T47D, and ZR-75-1 cell lines.

| *Apoptotic Regulators* | *MCF-7* | *T47D* | *ZR-75-1* |
|---|---|---|---|
| **Cell Surface Receptors** | | | |
| TNF, TNF-R | Expressed; stimulation results in PCD | Expressed; stimulation results in G1/S block | TNF expressed only when stimulated with exogenous TNF |
| TRAIL | Resistant | Resistant | Resistant |
| Fas,FasL | Resistant | Sensitive; FasL upregulated by Tam | Sensitive |
| **Bcl-2 family members** | | | |
| Bax | Expressed | Expressed; upregulated by antiFas Ab | Expressed |
| Bak | Downregulated by E2 | Expressed | |
| Bik | Induced by E2 deprivation and antiE2 | | |
| Bcl-2 | Upregulated by E2, downregulated by antiE2 | Undetectable basal expression; upregulated by E2 | Expressed; upregulated by E2 |
| Bcl-xL | Expressed | Expressed | Expressed |
| **Caspases** | | | |
| Caspase 3 | Negative | Expressed | Expressed |
| Caspase 7 | May substitute for caspase 3 | Expressed | Expressed |
| **p53** | wild type | Nonfunctional; Phe194 mutation | wild type |
| **PI3K/Akt** | Expressed; activation results in ER downregulation and antiE2 resistance | Expressed; Akt activation prevents Fas-mediated PCD | Expressed; activation results in ER downregulation |
| **NFκB** | Upregulated in antiE2-resistant variants | Expressed | Expressed |

Abbreviations: E2, estrogen; antiE2, antiestrogen; PCD, programmed cell death. See text for all other abbreviations and citations.

**Figure 1**. Antiestrogen effects on apoptotic signaling in breast cancer cells. Abbreviations: RTK, receptor tyrosine kinase; Mito., mitochondria. Other abbreviations have been defined in the text.